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14. ABSTRACT

The projects in this proposal specifically target several signal transduction pathways known to be critical for NSCLC pathogenesis including the EGFR pathway and the more downstream ras/raf/Mek/ERK pathway. These projects combine targeted approaches using molecular and imaging techniques to validate activity against a target and monitor response using imaging modalities specific to the receptor using either small molecules or targeted peptlde approaches.

The Developmental Research projects explore new areas including 1) the issue of high morbidity malignant pleural effusion thereby bringing the pulmonologists into the treatment of advanced disease with molecular therapies; and 2) prevention of lung cancer in youth through a highly interactive, entertaining CD-ROM program.

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TABLE OF CONTENTS

INTRODUCTION 2

BODY 2

- **Project 1** 2
- **Project 2** 8
- **Project 3** 12
- **Project 4** 19
- **Project 5** 26
- **Project 6** 29
- **Core B Biostatistics and Data Management** 36
- **Core C Molecular Pathology and Specimen Procurement** 38
- **Core D Molecular Imaging** 46
- **Developmental Research Project 1** 48
- **Developmental Research Project 2** 49
- **Career Development Project 1** 55

KEY RESEARCH ACCOMPLISHMENTS 57

REPORTABLE OUTCOMES 60

CONCLUSIONS 63

REFERENCES 65

APPENDICES 66

Appendix A (Flow Chart - Tissue Collection and Processing, Tarceva/Chemoradiation Trial, Project 1)

Appendix B (Project TALK Implementation Schedule)

Appendix C (Publications)

IMPACT: Imaging and **M**olecular Markers for **P**atients with **L**ung **C**ancer: **A**pproaches with Molecular Targets, **C**omplementary, Innovative and **T**herapeutic Modalities

INTRODUCTION

Lung cancer is the most prevalent cancer worldwide and the leading cause of cancer-related mortality in both men and women in the United States. Conventional multimodality therapies (surgery, radiation and chemotherapy) have reached a therapeutic ceiling in improving the five-year overall survival rate of non-small cell lung cancer (NSCLC) patients, clinically in large part due to chemo- and radiation-resistant locoregional and metastatic spread but ultimately due to poor understanding of the disease and its resistance to the therapy.

Lung cancer is a heterogeneous disease, resulting from accumulated genetic abnormalities over years, which thus requires a coordinated attack in a truly integrated fashion on multiple altered signal pathways. Emerging targeted therapy aims to target key molecular abnormalities in cancer and has succeeded in some tumor types such as chronic myeloid leukemia (CML) (Druker et al., 2004; Druker and Sawyers et al., 2001; Druker and Talpaz et al., 2001), gastrointestinal stromal tumor (Demetri et al., 2002), colon cancer (Hurwitz et al., 2003), and breast cancer (Howell et al., 2005). Thus, the incorporation of targeted therapy into conventional treatments appears to be a new promising approach to treatment of lung cancer.

The program project IMPACT has proposed to integrate targeted therapy in the lung cancer research program when initial clinical results showed disappointing response rates and survival benefit of epidermal growth factor receptor (EGFR) inhibitor Gefitinib (Iressa) for non-selected lung cancer patients (Herbst et al., 2002, 2003,2004; Herbst, 2004; Kris et al., 2003; Giaccone et al., 2004). It aims to validate molecular mechanisms of targeted agents alone and in combination with chemo and/or radiation therapies in preclinical and clinical settings. It also aims to develop effective molecular imaging and cancer cell-targeted peptide-based delivery tools to help improve efficacy of the targeted agents. Specifically, our objectives are:

- To validate preclinically and clinically several key signaling pathways and their agents for therapeutic potentials alone or in combination with each other or with chemo and /or radiotherapy
- To explore applications of molecular imaging for targeted therapy and identify cancer cell-targeted peptides for systemic delivery of therapeutic and imaging agents
- To discover and evaluate new molecular abnormalities and therapeutic predictors in lung cancer
- To develop an educational program for teens and young adults for smoking risk and resultant lung cancer occurrence.

IMPACT is composed of 6 research projects, 1 Biostatistics Core, 1 Molecular Pathology Core, 1 Molecular Imaging Core, 2 career development projects, and 2 developmental research projects. Here we present their scientific progresses in the third grant year as follows.

Project 1: Targeting epidermal growth factor receptor signaling to enhance response of lung cancer to therapeutic radiation.

(PI and co-PI: Raymond E. Meyn, Ph.D., Ritsuko Komaki, M.D.)

In spite of significant technical advances including intensity-modulated radiation therapy (IMRT) and chemoradiation, locally advanced lung cancer continues to have a dismal prognosis as many patients' tumors appear to be resistant to radiation therapy. The molecular basis for radiation resistance is not fully understood, but tumor cells have an enhanced survival response that involves increased capacity for DNA repair and suppressed apoptosis. Both apoptosis propensity and DNA repair capacity are thought to be partly controlled by the upstream signal transduction pathways triggered by EGFR activation, which is constitutively activated in many NSCLCs, and its activation leads to a radiation-resistant phenotype. We hypothesize that the response of NSCLC to radiation can be improved through the use of inhibitors of EGFR signaling.

Aim 1 To test the combination of external beam radiation and the selective EGFR-tyrosine kinase inhibitor erlotinib (Tarceva) in locally advanced NSCLC.

Update

A Phase II Study of Tarceva (erlotinib) in Combination with Chemoradiation in Patients with Stage IIIA/B Non-Small Cell Lung Cancer (NSCLC) has completed a comprehensive review by our Institutional Review Board (IRB), the US Department of Defense (DoD), Genentech, and Food and Drug Administration (FDA), and was finally approved during this past funding period. The trial was subsequently activated (11/20/07) and patient accrual has begun. This trial uses molecular targeted treatment (erlotinib, targeting EGFR) with chemoradiotherapy for stage III NSCLC to improve the therapeutic ratio (i.e., increase malignant cell cytotoxicity without increasing normal cell cytotoxicity). The primary objective is to determine the efficacy of concurrent erlotinib and chemoradiation as measured y time to progression. Secondary objectives include determining: 1) safety as measured by the rate of grade 3 or worse non-hematological toxicity (dose limiting toxicity, DLT) occurring prior to the beginning of consolidation therapy (including all toxicities attributed to chemoradiation occurring within 90 days of the start of radiation therapy); 2) compliance which is defined to be completion of concurrent chemoradiation plus erlotinib with no more than minor variations; 3) response rate (complete and partial response rates); 4) overall survival rates (one and two year rates, median survival); 5) disease local control rate; 6) association between EFGR expression and toxicity, response, overall survival, and progression (exploratory analysis); 7) association between EGFR expression and response correlated with imaging study. 72 patients will be treated with concurrent chemoradiation (RT: 63 Gy/35 fractions/7weeks (+/- 5 days), 1.8 Gy/ fraction, a total dose of 63.0 Gy in 35 fractions over 7 weeks plus Paclitaxel, 45mg/m^2, and Carboplatin, AUC=2, weekly for 7 weeks) and Erlotinib (Erlotinib, 150 mg p.o. daily for 7 weeks, starting with chemotherapy on day 2) followed by consolidation therapy of chemotherapy and Erlotinib (Weeks 11-17: Paclitaxel, 200 mg/m^2, and Carboplatin, AUC=6, every 3 weeks for two cycles).

Currently, 2 patients have been registered. However, one patient decided to go back home because of expenses to stay in Houston. The second patient had a borderline performance status and the medical oncologist decided concurrent chemotherapy could not be given safely. At present time, there are two patients pending for accrual to this protocol. Recent discussions have considered including the MDACC radiation treatment satellite clinics to optimize accrual and convenience to the patients.

Aim 2 To test the hypothesis that activation of the EGFR pathway leads to radiation resistance in NSCLC cells due to an enhanced capacity for repairing DNA lesions.

Studies conducted in this aim involved characterizing the signaling pathways downstream of EGFR activation in NSCLC cell lines and correlating radiation response and DNA repair capacity in the cell lines with their respective activation of EGFR.

Update

As reported last year, we have extended our investigations to include erlotinib to coincide with the ongoing clinical trial of this EGFR inhibitor plus radiation for the treatment of lung cancer in Aim 1 of this project. Thus, for the past 2 years, we have been studying the effects of erlotinib in parallel with completing our investigations of gefitinib.

As part of our continuing investigation into the mechanism responsible for erlotinib-mediated radiosensitization, we examined the effect of erlotinib treatment on the expression of downstream targets in the EGFR signaling pathway. As we reported last year, dose-dependent decreases in the levels of phosphorylated EGFR (pEGFR) are observed in unirradiated A549 and H1299 cells following 24-hour treatment with erlotinib. Moreover, we saw that radiation activates pEGFR in these cell lines but this activation is suppressed by erlotinib in a manner similar to what we have seen with gefitinib. As we originally predicted, the key to understanding tumor cell radioresistance due to an abnormal activation of EGFR may lie in the signaling pathways downstream of this receptor. In the case of gefitinib, we have shown that pERK is activated downstream of EGFR activation following irradiation and that this pERK activation is suppressed by gefitinib. We have followed up this observation by repeating this analysis for the case of erlotinib. As shown in Figure 1, radiation induces the activation of pERK within 2 hrs following irradiation in 2 NSCLC cell lines, A549 and H1299 (5 Gy). However, if the cells have been pretreated for 24 hrs with erlotinib, this activation is suppressed. Similar to what we have shown for gefitinib, we hypothesize that suppression of pERK activation downstream of EGFR by either gefitinib or erlotinib is the basis of radiosensitization by these agents.

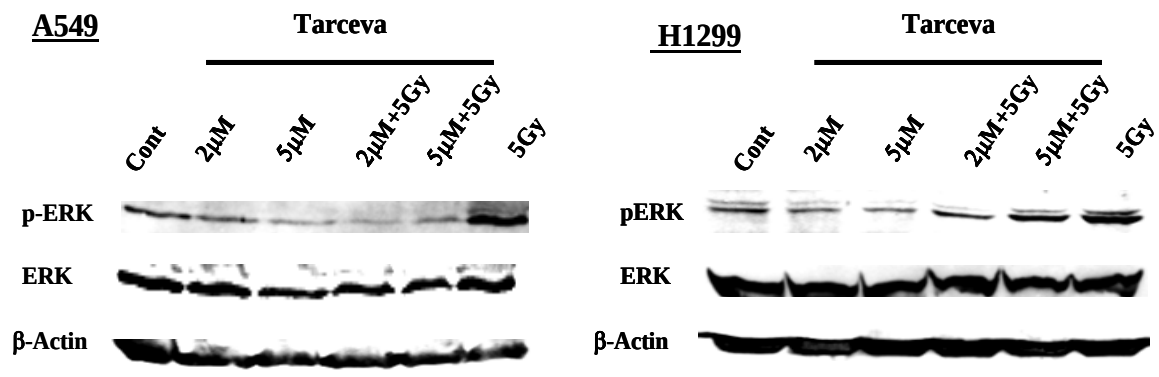


Figure 1. Western blot analysis of the effects of erlotinib (Tarceva) on pERK levels after irradiation. The effects of erlotinib treatment on pERK levels after irradiation for 2 hrs were analyzed by Western blot analysis in A549 and H1299 cell lines. Actin was used as a loading control.

Aim 3 To test the hypothesis that clinically useful inhibitors of EGFR signaling abrogate DNA repair capacity, restore apoptotic response and radiosensitize NSCLC cells.

Update

As mentioned last year, we have adopted two additional assays for the induction and repair of radiation-induced DNA double strand breaks (DSBs): the neutral comet assay and pulsed field gel electrophoresis (PFGE), to replace the host cell reactivation assay (HCR) used previously since these new assays are definitive for radiation-induce DSBs and superior to HCR. During this past year, we have used both of these assays to complete our paper on gefitinib recently published in *Clinical Cancer Research*.

As shown in Figure 2, for both cell lines and for both assays, a pretreatment with gefitinib did not, within experimental error, alter the initial level of DSBs induced when measured immediately after irradiation. However, again for both cell lines and both assays, this analysis confirmed that, in gefitinib-pretreated cells, DSB rejoining is delayed during the first 2 hrs after irradiation. In every case, the level of DSBs were higher than controls in the gefitinib-pretreated cells after 1 and 2 hrs of repair, and these differences were statistically significant at the $p < 0.05$ level.

A low level of residual unrejoined DSBs could be detected 24 hrs after irradiation in some instances, and although these seemed to be enhanced by gefitinib, these differences did not reach statistical significance. Nonetheless, these data are also consistent with gefitinib's ability to radiosensitize by suppressing cellular DSB repair capacity.

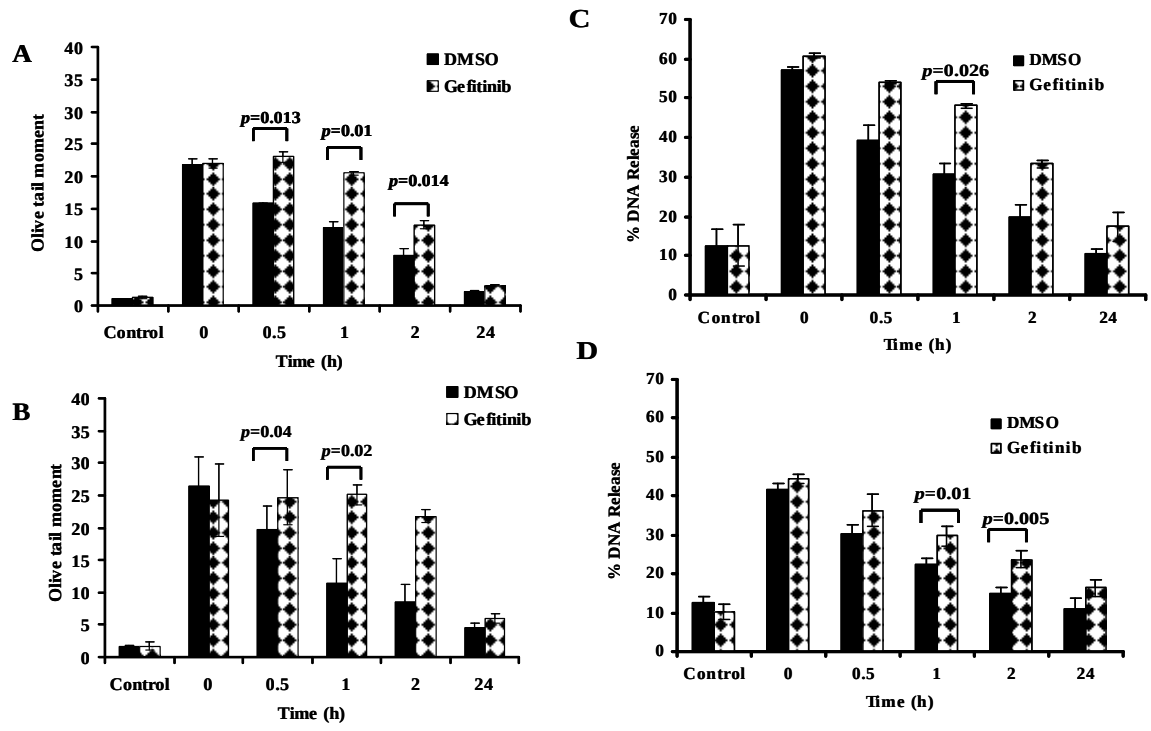


Figure 2. Gefitinib suppresses the repair of radiation-induced DSBs detected on the basis of the comet assay and PFGE. A549 (A and C) and H1299 cells (B and D) were exposed to gefitinib (1 M) for 24 h, irradiated with either 20 Gy for the comet assay (A and B) or 40 Gy for PFGE (C and D) and harvested at the indicated times. Each data point represents the average of three independent experiments. Bar: SE.

Additional experiments were carried out in an attempt to uncover what aspect of molecular repair is altered in gefitinib-treated cells. Because of the relative importance of radiation-induced activation of DNA protein kinases (DNA-PK) and ataxia-telangiectasia, mutated (ATM) in the induction of DSB repair, activation of these repair proteins was assessed. Gefitinib pretreatment did not alter the radiation-induced activation of these proteins (Figure 3A). Moreover, because our analysis of pNBS1 (phosphorylated Nijmegen breakage syndrome protein, also known as nibrin) foci indicated that the kinetics of activation of this protein may be altered in gefitinib-treated cells (data not shown), we assessed the activation of pNBS1 using immunoblot analysis. As shown in Figure 3B, for both cell lines, gefitinib enhanced the robustness and prolonged the pNBS1 signal after irradiation, confirming what was seen at the level of pNBS1 foci.

We speculate that because NBS1 functions in a protein complex, an abnormal abundance of NBS1 may sequester other repair proteins away from the sites of DSBs. At present there is no obvious connection between any of the signaling pathways downstream of EGFR and pNBS1. However, because NBS1 is a substrate for the ATM kinase, a possible connection between EGFR>ERK>ATM>NBS1 should be examined in further studies.

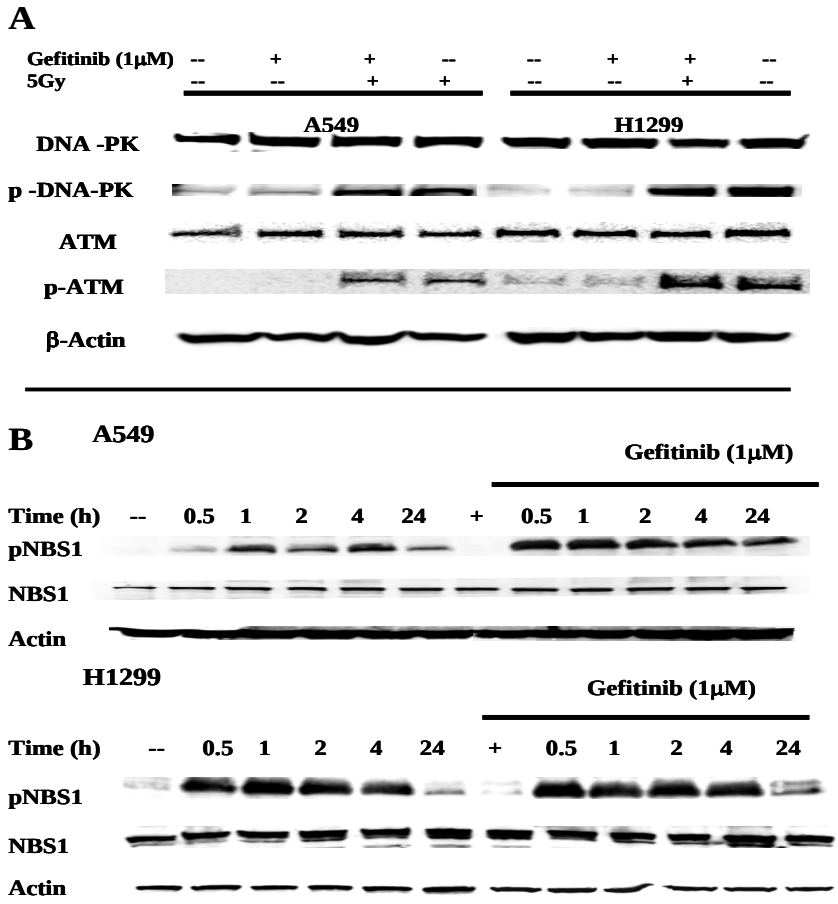


Figure 3. Gefitinib does not affect the nuclear levels of DNA-PK, ATM or their activated forms after irradiation but does enhance the activation of NBS1. A549 and H1299 cells were exposed to gefitinib (1 μM) for 24 h, irradiated, or not, with 5 Gy, and harvested 30 min after irradiation for the results in panel A or at the indicated times for panel B. Actin served as a loading control.

Aim 4 To test the hypothesis that targeting both EGFR and its downstream signaling pathways will have at least an additive radiosensitizing effect on NSCLC.

Update

We had previously begun to evaluate molecularly targeted agents that are designed to specifically target the Ras-Raf-Mek-ERK pathway downstream of EGFR. Testing of one such agent, sorafenib (BAY-43-9006), that targets Raf-kinase, has already been initiated and its radiosensitization of H1299 cells was reported last year. We have extended this analysis to

other small molecule inhibitors. It is now understood that resistance to EGFR inhibitors such as gefitinib and erlotinib may be due to compensatory signaling pathways. Two such pathways are now known: the c-Met and IGF1R mediated pathways. During this past funding period, we have begun to examine both of these pathways in order to understand why some cell lines are resistant to radiosensitization by inhibition of EGFR. Fortunately, there are commercially available inhibitors of c-Met (e.g., SU11274) and of IGF1R (e.g., AG1024). Thus, we have examined the ability of both of inhibitors to radiosensitize NSCLC cell lines. These studies are in progress but a preliminary analysis indicates that the c-Met inhibitor, SU11274, does have a significant radiosensitizing effect on H1299 cells as shown in Figure 4. This finding is consistent with the emerging hypothesis that resistance to radiosensitization with EGFR inhibition may be due to compensatory signaling by the c-Met receptor and that inhibition of c-Met signaling may be a useful strategy for radiosensitizing NSCLC cells either with or without combining with EGFR inhibitors.

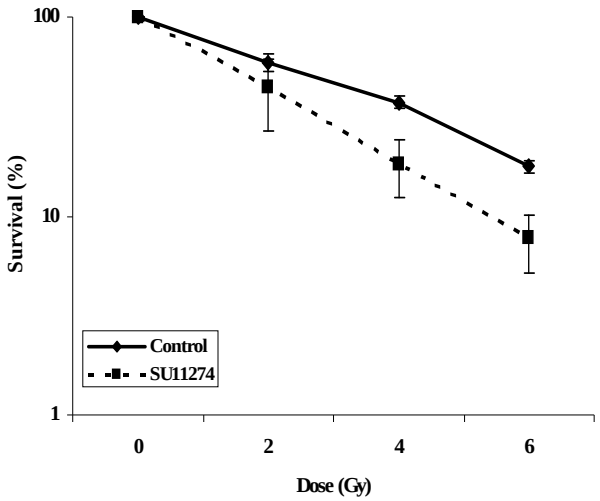


Figure 4. Radiosensitization by SU11274. H1299 cells were treated with 5μM SU11274 for 24hrs following which they were irradiated and plated for cell survival. Each data point represents average of 3 independent experiments plated in triplicate. Bar: SE.

Aim 5 To test whether the strategies developed in Specific Aims 2-4 have efficacy in a xenograft tumor model.

Update

This animal study was proposed for years 3 and 4. We prepared and submitted the animal protocol for review by our Animal Care and Use Committee (IACUC) which was approved on February 20, 2006. The approval letter and the animal protocol were included in last year's report. The animal experiments commenced during this past funding period. The experiments are currently still in progress.

Key Research Accomplishments

- Completed regulatory review of the clinical protocol for erlotinib plus chemoradiation which was approved by all agencies. This trial has been activated and patient accrual has begun.
- Demonstrated that, similar to the case of gefitinib, erlotinib also suppresses radiation-induced activation of pERK downstream of EGFR.
- Completed our investigation, using the neutral comet assay and pulsed field gel electrophoresis, showing that gefitinib suppresses the repair of radiation-induced DNA double strand breaks in NSCLC cells and submitted our findings for publication.
- Demonstrated that gefitinib may radiosensitize by inducing cells to express pNBS1 abnormally.

- Showed that the c-Met inhibitor, SU11274, radiosensitizes NSCLC cells presumably by suppressing the compensatory signaling that is responsible for resistance to radiosensitization by gefitinib.
- Initiated animal studies.

Reportable Outcomes

Manuscripts published in peer-reviewed journals

1. Toshimitsu Tanaka, Anupama Munshi, Colin Brooks, Jenny Liu, Marvette L. Hobbs, and Raymond E. Meyn. Gefitinib Radiosensitizes Non-Small Cell Lung Cancer Cells by Suppressing Cellular DNA Repair Capacity. Clin Cancer Res 14(4)1266-73, 2008.

Conclusions

Based on the work conducted to date, we are building a model to explain the molecular mechanism by which small molecule, tyrosine kinase inhibitors such as gefitinib and erlotinib suppress the cellular capacity for repair of DSBs thereby radiosensitizing NSCLC cells. Resistance to this mechanism may be due to compensatory pathways and these pathways can also be targeted with novel agents to restore radiosensitivity. Now that the animal studies and the clinical trial are underway, we will be able to validate that these same mechanisms are active and that these strategies are efficacious *in vivo*.

Project 2: Molecular Imaging of EGFR Expression and Activity in Targeting Therapy of Lung Cancer

(PI and co-PI: Juri Gelovani, M.D.; Roy Herbst, M.D., Ph.D.)

As originally planned, during the third year of funding of Project, our research continued to be focused on Aims 2 and 3 of this Project.

Aim 1 To synthesize novel pharmacokinetically optimized ^{124}I and ^{18}F-labeled IPQA derivatives for PET imaging of EGFR kinase activity and conduct *in vitro* radiotracer accumulation studies in tumor cells expressing different levels of EGFR activity.

No additional progress was made in the second year on this Aim.

Aim 2 To assess the biodistribution (PK/PD) and tumor targeting by novel ^{124}I and ^{18}F-labeled EGFR kinase-specific IPQA derivatives using PET imaging in orthotopic mouse models of lung cancer and compare *in vivo* radiotracer uptake/retention with phospho-EGFR levels *in situ*.

As part of Aim 2, we developed the fourth-generation of water-soluble polyethylene glycol (PEG)-ylated 3-iodo-4-(phenylamino)quinazoline-6-acrylamide (IPQA) derivatives labeled with ^{18}F, which will simplify translation of EGFR kinase imaging agent into the clinic in comparison with ^{124}I-JGAP5. These novel fourth generation ^{18}F-labeled compounds were synthesized by the Imaging Core D (Figure 5). It is noteworthy, that compound ^{18}F-PEG6-IPQA was developed

based on our previous studies, because it was highly is soluble in water and was expected to have less hepato-biliary clearance and a longer plasma circulation half-time.

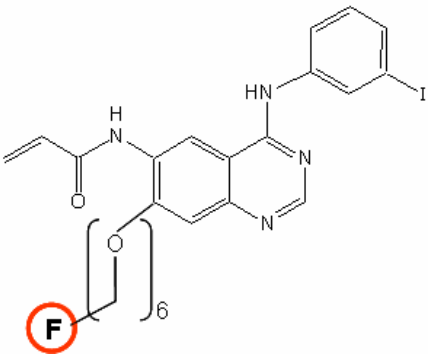


Figure 5. Structure of a novel IV-generation agent ^{18}F-PEG6-IPQA with improved selectivity and water solubility.

Initially, the ^{18}F-PEG6-IPQA was assessed *in vitro* for accumulation and washout kinetics in human NSCLC cells with different EGFR signaling profiles (PC14, H441, H3255, H1975) (Figure 6). The accumulation of ^{18}F-PEG6-IPQA *in vitro* in most of these NSCLC cells was higher than that previously observed with radio-iodinated JGAP5 (third-generation compound). The most progressive accumulation and the slowest washout was observed in H3255 expressing dominant active mutant L858R EGFR kinase. In contrast, no progressive accumulation and almost complete washout (down to an equilibrium level of 1) was observed in H411 cells that overexpress TGFα and EGFR (positive autocrine loop), in PC14 with EGFR-independent growth (low EGFR) and H1975 with dual mutation in EGFR kinase domain that precludes ^{18}F-PEG6-IPQA from binding and covalent adduct formation. Further studies demonstrated that the accumulation of ^{18}F-PEG6-IPQA at 60 to 120 min in these cell lines was inhibited by pre-treatment with Iressa in a dose-dependent manner (Figure 6). The latter was especially dramatic in H3255 cells expressing dominant active mutant EGFR kinase. Therefore, these *in vitro* radiotracer uptake and washout studies have demonstrated the highest uptake and retention of ^{18}F-PEG6-IPQA in H3255 NSCLC cells with increased activity of dominant active mutant EGFR kinase and high sensitivity to therapy with Iressa.

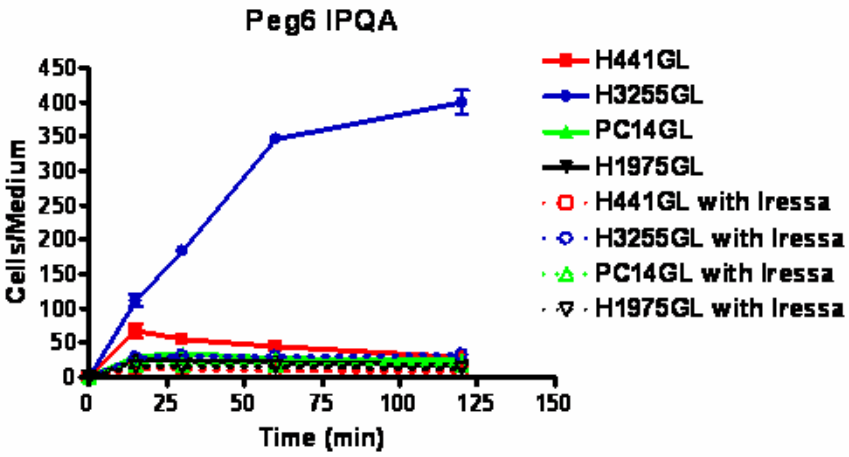


Figure 6. Time-dependent accumulation and washout of ^{18}F-PEG6-IPQA in different NSCLC cell lines. The accumulation of ^{18}F-PEG6-IPQA in these cell lines was inhibited by pre-treatment with Iressa in a dose-dependent manner. A significantly increased (~40 fold) accumulation of ^{18}F-PEG6-IPQA was observed at 60 to 120 min in H3255 NSCLC cells that express dominant-active mutant L858R EGFR, as compared to the III-generation compound JGAP5.

Aim 3 **Using selected ^{124}I or ^{18}F-labeled IPQA derivative, to conduct pre-clinical studies in animals with orthotopic models of lung cancer xenografts with different levels of EGFR expression/activity, and to assess the value of PET imaging as the inclusion criterion for therapy by EGFR inhibitors, as well as for monitoring the efficacy of treatment with EGFR-targeted drugs.**

As part of Aim 3, we have initiated *in vivo* PET imaging studies with ^{18}F-PEG6-IPQA in mice bearing s.c. xenografts of four human NSCLC cells that express TGFα at different levels and express either the wild-type or mutant EGFR kinases (PC14, H441, H3255, H1975). As reported previously, these cell lines have been retrovirally transduced with *eGFP – Firefly luciferase* fusion reporter gene (GL) to facilitate non-invasive imaging of s.c. and orthotopic NSCLC xenografts in mice. The GL reporter gene allows for fluorescence microscopic and FACS analysis of GL-expressing cells *in vitro* and bioluminescence imaging (BLI) of GL-expressing tumor xenografts in mice *in vivo*. PET and autoradiography demonstrated that H3255 tumors accumulated significantly higher levels of ^{18}F-PEG6-IPQA (Figure 7; PET and autoradiography/ visible panels) as compared to [^{124}I]JGAP5 and [^{124}I]mIPQA ($p<0.05$) in our previous studies (years 2 and 3). The accumulation of ^{18}F-PEG6-IPQA was also observed in hair follicles in the skin in lungs and bronchial epithelium, which serve as an "internal control tissue". In general, PET imaging of ^{18}F-PEG6-IPQA performed at 3 hours after intravenous (i.v.) administration

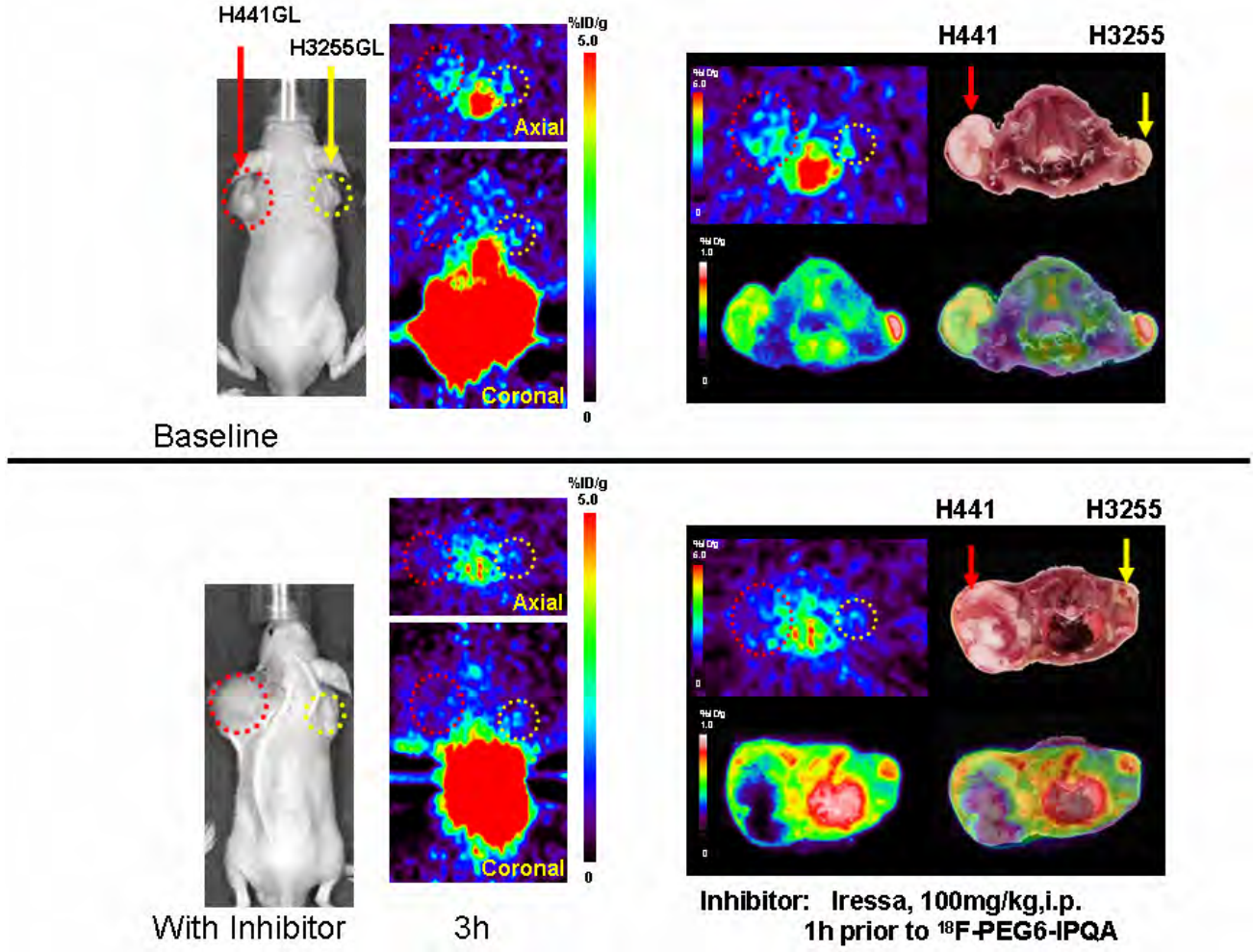


Figure 7. PET imaging and autoradiography of H3255 and H441GL s.c. tumors in mice obtained at 3 hours after i.v. administration of ^{18}F-PEG6-IPQA (150 uCi/animal). The autoradiographic images were obtained from the whole body axial cryosections of mice through the bulk of the tumor xenograft and spatially co-registered with the visible image of and GFP-fluorescence image of the same section. The highest level of radioactivity accumulation was observed in H3255 tumors, expressing dominant-active mutant L858R EGFR kinase. Moderate-to-high levels of accumulation of this agent was also observed in viable areas of H441 tumors that over-express wild-type EGFR and TGFα.

demonstrated substantial accumulation of ^{18}F-PEG6-IPQA-derived radioactivity in H3255 and moderate-to-high levels in H441 tumors. In contrast, significantly lower levels of ^{18}F-PEG6-IPQA accumulation were observed in PC14 and H1975 tumors (data not shown due to space limitations). The later is due to low level of EGFR expression-activity in PC14 cells and the presence of dual mutation in EGFR kinase domain of H1975 cells (L585R activating mutation and T790M mutation which precludes ^{18}F-PEG6-IPQA from binding to the ATP binding site).

Co-registration of autoradiographic images with corresponding anatomical images of the same sections that were used to produce autoradiograms, provided high resolution images that explain the pattern of ^{18}F-PEG6-IPQA distribution at 3 hours post i.v. administration. Such pattern could not be visualized and analyzed by microPET, because it has a much lower resolution and sensitivity than autoradiography.

The total accumulation of ^{18}F-PEG6-IPQA was significantly decreased by pre-treatment of the mice with Iressa (100 mg/kg i.p.), as compared to non-treated control animals ($p<0.01$) (Figure 7). This observation clearly demonstrates the feasibility of PET/CT imaging with ^{18}F-PEG6-IPQA for prediction of tumor responsiveness to therapy with EGFR kinase-targeted inhibitors and for the assessment of biologically effective (adequate) dose for treatment of individual tumors (and maybe applicable in patients).

Ongoing Studies. Based on the requirements of the FDA for pre-clinical characterization of novel radiolabeled compounds for diagnostic imaging, we are currently conducting PET imaging studies with [^{18}F]-PEG6-IPQA in non-human primates for the assessment of PK, biodistribution, metabolism, and radiation dosimetry. These studies are required for preparation of an Investigational New Drug (IND) application.

In parallel, we have contracted Charles River Laboratories to conduct acute toxicity studies in mice with non-radiolabeled (cold) F-PEG6-IPQA at 100x the pharmacologic dose equivalent (per kg) of the radiolabeled dose of [^{18}F]-PEG6-IPQA, that will be used in humans. These studies are required for preparation of an IND.

Also, we are optimizing the radiolabeling of [^{18}F]-PEG6-IPQA and developing chemistry, manufacturing, and control (CMC) section of an IND protocol.

We have sub-contracted CGMP synthesis of a “cold” precursor for radiolabeling of [^{18}F]-PEG6-IPQA from Macrocyclics Inc. (Dallas, TX), which will be used in future Phase I clinical trial.

Key Research Accomplishments

- It is noteworthy, that the hepatobilliary clearance of the ^{18}F-PEG6-IPQA radioactivity was further reduced as compared to the previous two generations of compounds - [^{124}I]-JGAP5 and [^{124}I]-mIPQA, which explains (at least in part) the reason for higher accumulation of water-soluble [^{18}F]-PEG6-IPQA in tumor tissue, which is due to increased clearance half-time and the AUC (input function) in blood.
- A significantly higher tumor-to-lung accumulation ratio (>10-fold contrast) was observed at 24 hours after i.v. administration of ^{18}F-PEG6-IPQA, which was higher than that observed with [^{124}I]-mIPQA (about 6-7 fold).
- Most importantly, we have developed a novel agent which is highly selective for L858R dominant active mutant EGFR and can provide highly specific PET imaging results.
- Pre-IND pharmacokinetics and radiation dosimetry studies for [^{18}F]-PEG6-IPQA are underway in non-human primates.
- Pre-IND acute toxicology studies are underway and are subcontracted to a company (Charles River Labs).

- CGMP production of precursor for ^{18}F-radiolabeling of [^{18}F]-PEG6-IPQA has been subcontracted to CGMP qualified company (Macrocyclics, Inc., Dallas, TX).

Reportable Outcomes

Abstracts

1. Nishii R, Pal A, Soghomonyan S, Balatoni J, Mushkudiani I, Yeh HH, Mukhopadhyay U, Volgin A, Shavrin A, Maxwell D, Tong W, Alauddin M, Bornmann W, Gelovani J. PET Imaging of Different EGFR Kinase Mutant NSCL Carcinomas with [^{18}F]-PEG6-IPQA for Prediction of Responsiveness to EGFR Kinase Inhibitors. Proceedings of the 5th Annual Meeting of the Society of Molecular Imaging, Providence, September 6-9, 2007.

Conclusions

Several important findings have led us to the pre-IND phase with our lead compound identified. The [^{18}F]-PEG6-IPQA compound so far is the most PK optimized compound worthy of clinical translation. Imaging with pharmacokinetically optimized more water-soluble [^{18}F]-PEG6-IPQA (as opposed to [^{124}I]-mIPQA and [^{124}I]JGAP5 compounds) should allow for identification of tumors with increased EGFR signaling. The accumulation of [^{18}F]-PEG6-IPQA is highest in H3255 NSCLC cells that express L585R active mutant EGFR and correlates positively with the sensitivity of tumors to EGFR inhibitors (and is better than with [^{124}I]-mIPQA and [^{124}I]JGAP5). In addition, the accumulation of [^{18}F]-PEG6-IPQA can be observed to some extent in normal tissues that express EGFR (i.e., hair follicle cells), which are currently used as surrogate biomarkers of EGFR activity/inhibition and which represents additional proof of the approach to imaging EGFR kinase activity with [^{18}F]-PEG6-IPQA.

Project 3: Targeted Peptide-based Systemic Delivery of Therapeutic and Imaging Agents to Lung Cancer

(PI and co-PI: Renata Pasqualini, Ph.D., Wadih Arap, M.D., Ph.D.)

The studies outlined in this proposal focus on the use of peptide sequences with selective lung tumor targeting properties. We will seek to validate these probes as delivery vehicles in drug and gene targeting approaches. This approach directly selects *in vivo* for circulating probes capable of preferential homing into tumors. The strategy will be to combine homing peptides in the context of phage as gene therapy vectors. Given that many of our peptides also target angiogenic vasculature in addition to tumor cells, these studies are likely to enhance the effectiveness of therapeutic apoptosis induction and imaging technology.

Aim 1 To select peptides targeting primary and metastatic tumors in lung cancer patients.

Update

Pre-clinical studies for pre-IND application package: BMTP-11 and BMTP-78. We have two GMP grade drugs geared for IND filing with the FDA. They are targeted to the interleukin 11 receptor (IL-11R) and to GRP78. Dr. Wistuba (Director, Core C) has performed extensive studies in human lung cancer tissue samples. These studies indicate that these targets are

suitable for delivery of therapies and imaging agents to lung cancer. Extensive toxicology work has been completed in mice and rats. Efficacy studies in lung cancer model are ongoing. Primate (Cynomologous monkey) studies have been performed and an extensive set of reports compiled for a pre-IND package focused on BMTP-11, a proapototic peptide targeted to IL-11R. A clinical protocol for a Phase I trial in metastatic lung cancer is in preparation, and pre-clinical data package is being completed with mouse, rat, and non-human primate toxicology and mouse efficacy using 6 tumor models.

Aim 2 To validate receptors for targeting human lung cancer.

Update

As reported previously, we identified the lung cancer-targeting receptor EphA5 and validated ephrin-mimic peptides (CSGIGSGGC and CRFESSGGC) in lung cancer.

EphA5 Receptor in Lung Cancer. As discussed, this project is based on the discovery of an entirely novel ligand-receptor pair in lung cancer. The targeting peptide and subsequent receptor were identified in the NCI 60 cell surface fingerprinting by phage display (Kolonin et al., Cancer Research, 2006). We are now performing additional experiments with phage and peptide targeting. Important optimization has been accomplished in terms of *in vitro* experiments and the experiments are being repeated to reach publication quality. Deciphering the mechanisms underlying the over-expression of EphA5 in lung cancer will be of interest. We believe we already know which pathways are being affected and the proteins involved but we need more time to complete state-of-the-art cell biology and biochemistry work to confirm. We are searching for the best model as far as which cell lines to use as proof-of-principle. As for the *in vivo* part of this project, we have made progress (see below); our data demonstrate that the phage homing to subcutaneous tumors is promising for one of the peptides. We are continuing efforts to optimize the orthotropic model and prepare for therapeutic trials.

CSGIGSGGC and CRFESSGGC synthetic peptides specifically inhibit phage binding to EphA5. There are two phage being validated in this project, both displaying peptides with the SSG motif that was selected first based on binding to lung cancer cell lines (NCI-60) and second on *in vitro* selection on immobilized EphA5. Homology search to possible ligands along with the data we generated *in vitro* suggest that ephrin B3 is the protein mimicked by the peptides. Ephrin A4 is also being considered but it is expected that this protein binds to EphA4 as well, which we do not see for the peptide *in vitro*. However, ephrin A4 will continue to be considered as a ligand at this time because there could be a site of interaction that discriminates between these two receptors. We tested the ability of the synthetic peptides CSGIGSGGC and CRFESSGGC to inhibit specific phage binding to EphA5. Figures 8A and 8B show the binding of CSGIGSGGC – phage to EphA5 in presence of increasing concentrations of CSGIGSGGC (a) and CRFESSGGC (b) peptides. Insertless phage and CARAC (Cys - Ala - Arg - Ala – Cys; an unrelated peptide used as a control for non-specific binding) were used as controls. Both peptides specifically inhibited phage binding, whereas the controls did not. Figures 8C and 8D show the binding of CRFESSGGC – phage in presence of each peptide. Again, insertless phage and CARAC were used as controls. In this case, inhibition with the CSGIGSGGC peptide was not complete, at least not in this range of peptide concentration. CRFESSGGC peptide abolished phage binding at the concentration of 1nM. These were all performed at the same time.

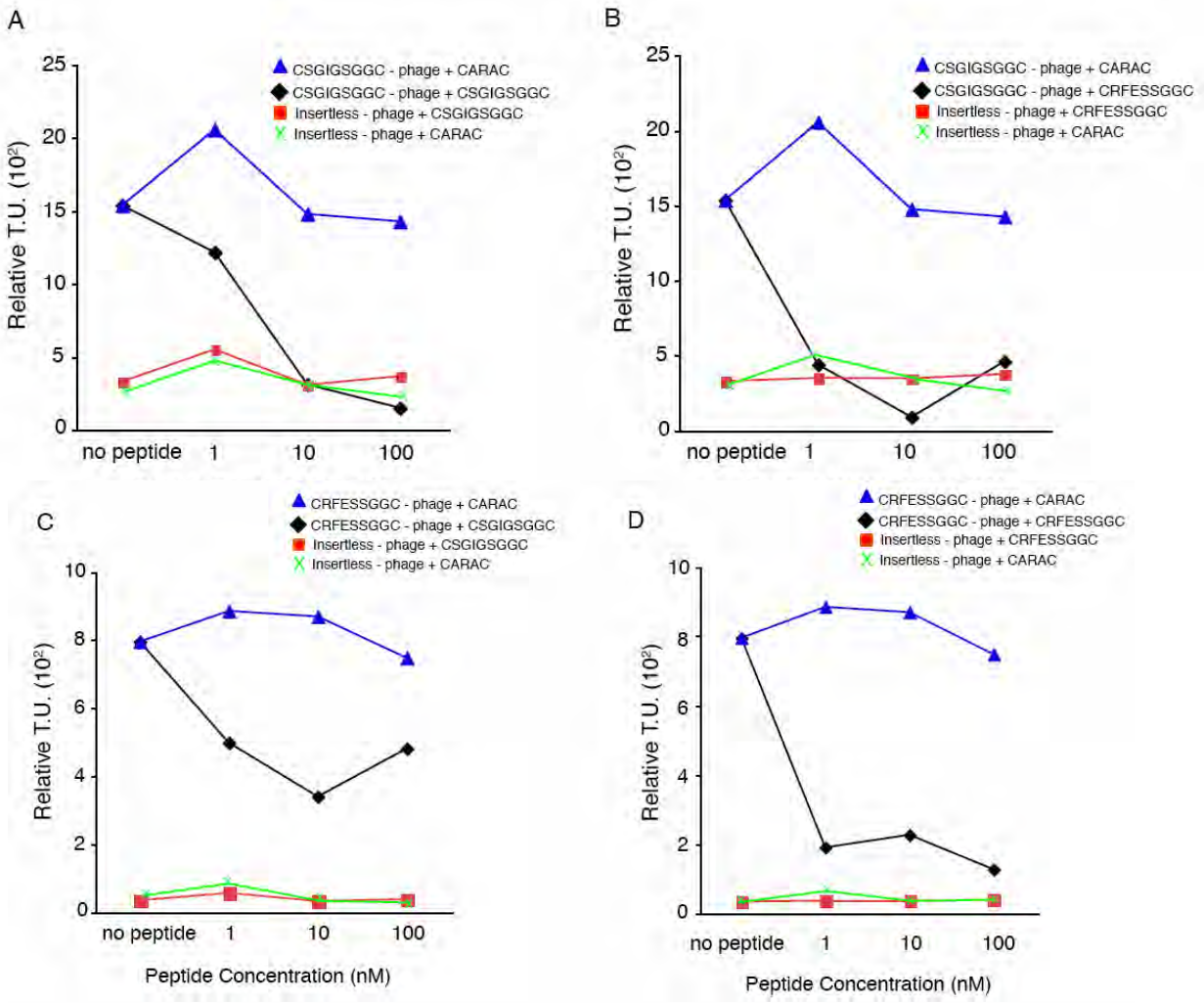


Figure 8. Phage binding inhibition by cognate peptides. Microtiter wells were coated with EphA5 receptor and 10^9 TU of phage were added in presence of different peptide concentrations. After incubation for 1 hr and several washes, bound phage were recovered by bacterial infection. (A and B) Inhibition of CSGIGSGGC-phage binding to the targeted receptor by the cognate peptide CSGIGSGGC (A) or the peptide CRFESSGGC (B). (C and D) Inhibition of CRFESSGGC-phage binding to EphA5 receptor in presence of (A) CSGIGSGGC or (B) CRFESSGGC synthetic peptides. Experiments were repeated a minimum of 3 times each.

Aside from phage binding, immunofluorescence (IF) and internalization assays need to be performed with the H460 and A549 lung cancer cells. It would be important to work with the same cells *in vitro* and *in vivo*, as expression of proteins might have caused us to fail when trying to establish the mouse model with our cells. Proliferation assays were performed (reported last year) and will serve as a guide for us in terms of concentration of peptide and incubation time to be used. In summary, we will find the most stable and reproducible conditions before pursuing signal transduction experiments and siRNA based constructs to produce null cells.

***In vivo* homing of CSGIGSGGC and CRFESSGGC – phage to subcutaneous Lewis Lung Cancer (LLC) cells.** Animals were injected subcutaneously with LLC cells; all developed tumors within 10 days after injection. Phage were intravenously administered at 10^{10}

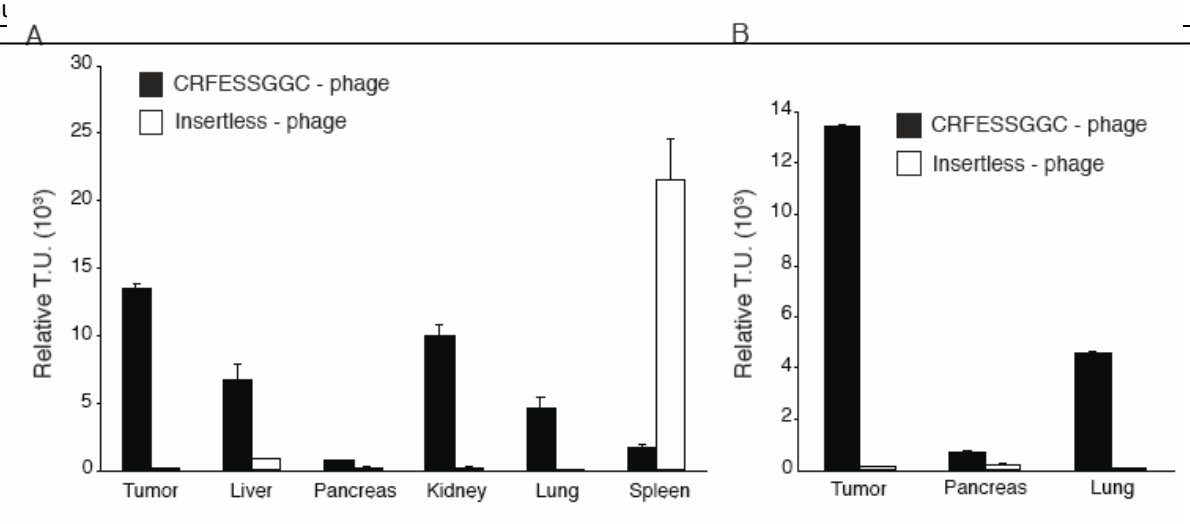


Figure 9. *In vivo* homing of targeted phage. (A) Homing of CRFESSGGC – phage to subcutaneous Lewis Lung Cancer compared to control organs (pancreas and normal lung). Specific homing of targeted phage to tumor is observed. (B) Phage were enriched by homing to tumor.

transforming units (TUs) for each clone. After 6 hours, animals were perfused and organs collected for phage recovery, immunohistochemistry (IHC) and IF analyses. Figure 9A shows homing of CRFESSGGC – phage to tumor compared to various organs. Figure 9B shows the plot representing enrichment (tumor homing), demonstrating apparent specificity.

Finally, injection of H460 successfully produced nodules in the lung of nude mice. Staining for phage and EphA5 on subcutaneous LLC tumors gave an impressive positive signal, mostly vascular (Figure 10). Targeted phage were also found outside vessels. Preliminary results from double staining with anti-phage and anti-EphA5 antibodies shows co-localization of phage and EphA5 (Figure 11). The orthotropic model will give us more specific results.

As described in the original grant proposal, we have identified circulating probes targeting the IL-11R and GRP78 receptors and evaluated them as a “beacon” for the delivery of anti-cancer drugs (Arap et al; Zurita et al, 2004). Our preliminary data showed that these proapoptotic peptides (Arap et al, 2004; Zurita et al, 2004) targeted to the receptors specifically induce apoptosis in lung cancer cell lines. We are confirming these results and will present the data in next year’s report.

In collaboration with Dr. Wistuba (Director, Core C), we examined the expression of GRP78, IL-11R and EphA5 in lung cancer tissue microarrays (TMAs) containing 301 NSCLCs (192 adenocarcinomas and 109 squamous cell carcinomas) and 26 SCLCs with annotated clinical data. We performed analysis of immunostaining using microscopic evaluation by 2 independent observers, and consensus data were used for further analysis. Overall, high levels of GRP78, IL-11R and EphA5 expression were detected in lung cancer tumor specimens. Significant differences in the expression of these markers were detected comparing both tumor types: a) GRP78 expression in NSCLCs histologies, adenocarcinoma and squamous cell carcinoma, was significantly higher in the cytoplasm and membrane of tumor cells compared with SCLC; b) IL-11R showed higher levels of cytoplasmic expression but lower levels of membrane expression in NSCLCs; c) EphA5 expression was higher in NSCLC tumor cells compared with SCLC.

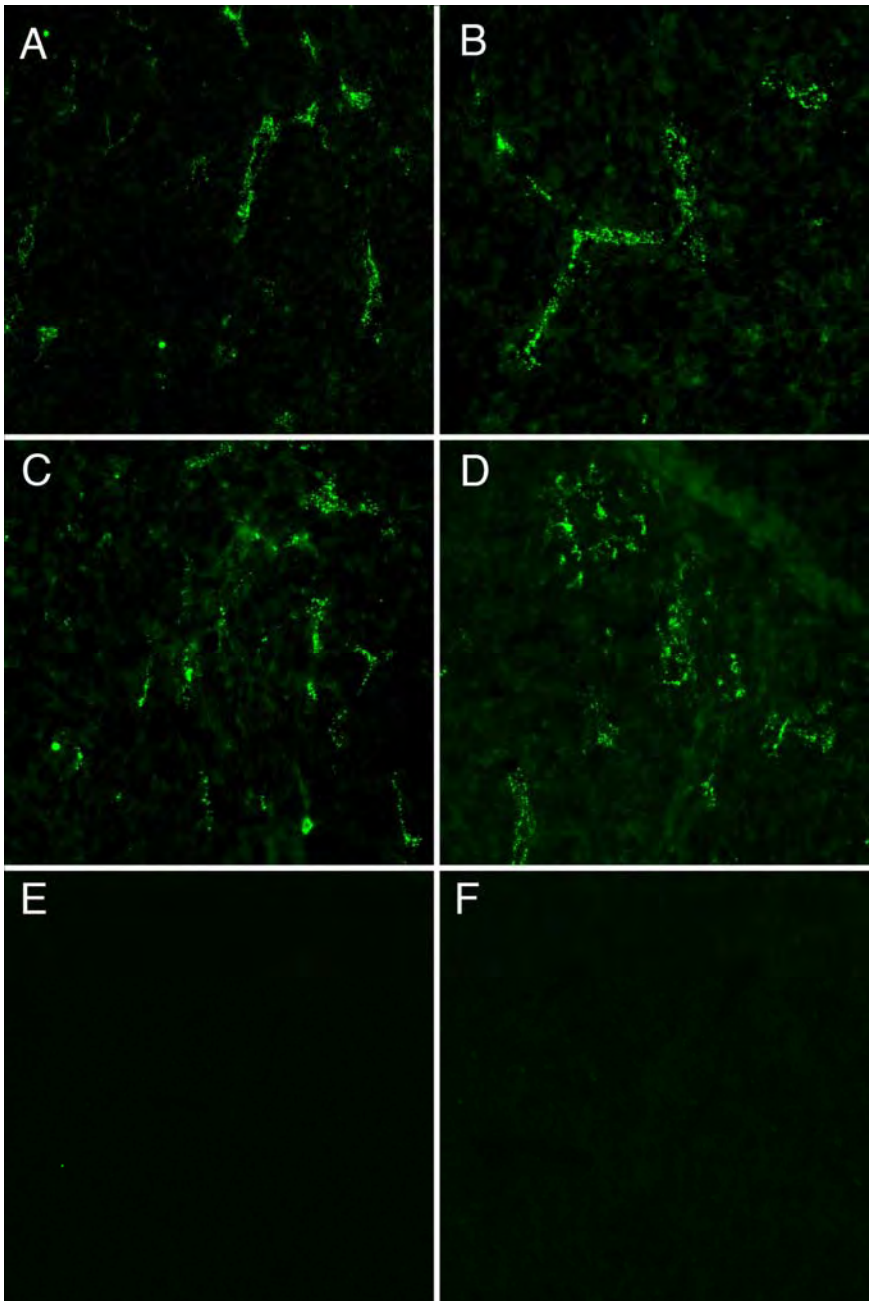


Figure 10. Phage staining on Lewis Lung carcinoma. A – D: Examples of phage staining in subcutaneous tumors. E: insertless phage. F: control antibody.

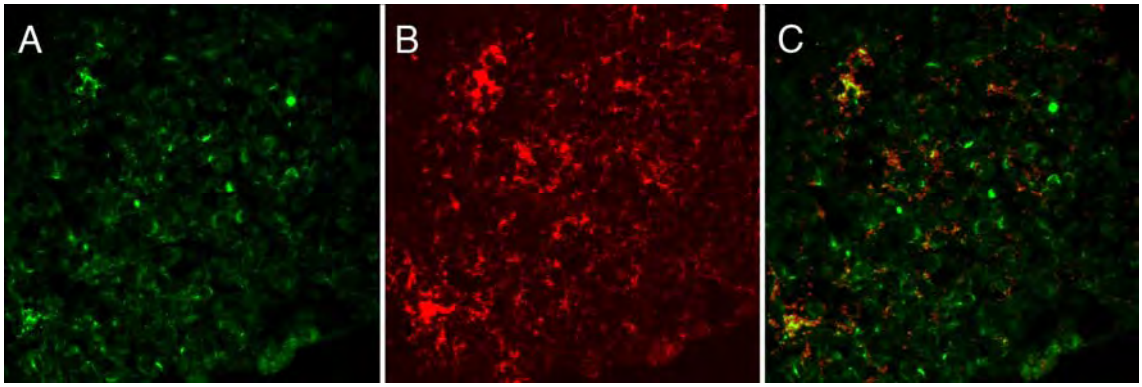


Figure 11. Co-localization of EphA5 (A) and phage (B) in subcutaneous LLC. (C) Merge of images A and B.

For NSCLC adenocarcinoma and squamous cell carcinoma histologies, a detailed analysis comparing GRP78, IL-11R and EphA5 expression in tumor cells with patients' clinicopathologic features, including tumor histology, age, gender, smoking history, pathological TNM stage, disease free and overall survival, was performed. Significant differences in the expression of markers were detected comparing both NSCLC histologies with adenocarcinomas demonstrating higher levels of cytoplasmic GRP78 (p = 0.0003) and cytoplasmic IL-11R (p < 0.0001). In contrast, squamous cell carcinomas showed significantly higher expression for membrane GRP78 (p = 0.003) and EphA5 (p = 0.002). Only IL-11R demonstrated correlation with smoking status, with tumors from ever smokers having higher levels (P = 0.005) of cytoplasmic IL-11R than never smokers. No correlation between marker expression and disease free and overall survivals was detected. For more detailed data of the IHC analysis, please refer to Aim 4 of Project 3 in the Molecular Pathology Core of this report.

These findings will be expanded as they provide rationale for proposing that these molecular receptor signatures within human tumors are suitable for targeted delivery of drugs.

Aim 3 To design tools for molecular imaging of lung tumors.

Update

We are in the process of evaluating the efficiency of targeted delivery of luciferase, green fluorescence protein (GFP) and thymidine kinase in lung cancer animal models, in collaboration of Dr. Juri Gelovani, the Director of the IMPACT Molecular Imaging Core. Any definitive results will be presented in the next report. A new phage-based vector with this triple reporter gene construct has been cloned. These new AAVP vectors, which are based on previously published work (Hajitou et al, Cell, 2006), not only have novel targeting peptides but also contain tumor specific promoters other than the original CMV promoter. The idea is to construct the lung cancer transcriptome based on ligand directed delivery and specific gene expression profiles.

Other promising receptor-ligand systems will be also leveraged for targeted imaging, such as vectors targeting EphA5, GRP78, and IL-11R, which have already been validated in patient derived samples as described above and in the Molecular Pathology Core report.

Key Research Accomplishments

- Demonstrated specificity of phage for EphA5 receptor.
- Determined that phage expressing these peptides selectively localize to tumors *in vivo*.
- Confirmed that, overall, high levels of GRP78, IL-11R and EphA5 expression were detected in lung cancer tumor specimens.
- Compiled a pre-IND package to be submitted to the FDA surrounding existing toxicology and efficacy data for a proapototic peptide targeting the IL-11R. This package includes extensive toxicology studies performed in non-human primates.

Reportable Outcomes

Resources

We are generating an extensive database for targeting ligands and vascular receptors identified in our laboratory. This database is likely to be very useful as it can be integrated with the system that is in place under the IMPACT Program to correlate clinical information and responses to therapy with the expression of selective molecular targets.

Manuscripts published in peer-reviewed Journals

1. Hajitou A, Rangel R, Trepel M, Soghomonyan S, Gelovani JG, Alauddin MM, Pasqualini R, Arap W. Design and construction of targeted AAVP vectors for mammalian cell transduction. Nat Protoc 3:523-31, 2007.

2. Rangel R, Sun Y, Guzman-Rojas L, Ozawa MG, Sun J, Giordano RJ, Van Pelt CS, Tinkey PT, Behringer RR, Sidman RL, Arap W, Pasqualini R. Impaired angiogenesis in aminopeptidase N-null mice. Proc Natl Acad Sci U S A 104:4588-93, 2007.

3. Jaalouk DE, Schlingemann RO, Ozawa MG, Sun J, Lahdenranta J, Pasqualini R, Arap W. The original PAL-E monoclonal antibody recognizes a VEGF-binding site within neuropilin-1. Cancer Res. In press.

Manuscripts in press

1. MG, Zurita A, Dias-Neto E, Nunes DN, Sidman RL, Gelovani JG, Arap W, Pasqualini R. Beyond Receptor Expression Levels: The Relevance of Target Accessibility in Ligand-directed Pharmacodelivery Systems. Trends in Cardiovascular Med, in press.

2. Hajitou A, Lev DC, Hannay JA, Staquicini FI, Soghomonyan S, Alauddin MM, Benjamin RS, Pollock RE, Gelovani JG, Pasqualini R, Arap W. Monitoring Therapeutic Drug Response in Sarcoma with Targeted AAVP Molecular-Genetic Imaging. PNAS, in press.

Conclusions

EphA5 protein overexpression in lung cancer cells, in light of candidate ephrin mimics (GGS peptides) targeting these cells, provides original evidence for EphA5 being a lung cancer marker and has potential functional implications. Next, we will setup the cell biology and biochemistry experiments for siRNA downregulation of EphA5. We will also use lentiviruses as an alternative approach. We will also test the circulating peptides for IL-11R and GRP78 *in vivo*, in lung cancer animal models, and evaluate their therapeutic properties in order to complete assembly of a pre-IND package related to a Phase I clinical trial in lung cancer patients (Dr. Roy Herbst will lead the clinical study). We also plan to image the effects of other targeted peptides delivered to

angiogenic vasculature using sophisticated vascular imaging technology. These studies should be highly informative and will shed light into the mechanistic aspects of the anti-tumor and anti-angiogenic activity in lung cancer. Novel AAVP constructs will be evaluated *in vivo*. These vectors will display different targeting peptides and lung cancer specific promoters. A therapeutic approach related to the delivery of TNF to tumor vasculature will be evaluated. RGD4C-AAVP-TNF has been evaluated successfully in tumor bearing pet dogs and a clinical trial is planned to begin as part of a collaboration with the NCI.

Project 4: Inhibition of bFGF Signaling for Lung Cancer Therapy

(PI: Reuben Lotan, Ph.D.)

The survival of lung cancer patients is poor because this cancer is diagnosed at advanced stages. Therefore, improvements in early detection through the identification of molecular markers for diagnosis and for intervention combined with targeted chemoprevention are urgently needed. While the molecular events involved in lung cancer pathogenesis are being unraveled by ongoing large scale genomics, proteomics, and metabolomics studies, it is already well recognized that proliferation-, survival- and angiogenesis- promoting signaling pathways are amplified in lung cancer. Among the angiogenesis signaling pathways, the basic fibroblast growth factor (bFGF) and its transmembrane tyrosine kinase receptors (FGFRs) are playing important roles in addition to the well-studied vascular endothelial growth factor (VEGF) and its receptors (VEGFRs). Both types of angiogenesis signaling pathways, the VEGF/VEGFR and the bFGF/FGFR, have been detected in NSCLC and associated with lung cancer development. However, most efforts in preclinical and clinical trials have been directed to the VEGF/VEGFR pathway.

We hypothesize that bFGF triggers signaling pathways that contribute to malignant progression of lung cancers by stimulating tumor cell and endothelial cell proliferation and survival and augmenting angiogenesis. Therefore, agents that intervene in this pathway may be useful for lung cancer therapy either alone or in combination with agents that target the VEGF/VEGFR signaling pathways and/or with cytotoxic agents. We will address the following specific aims in order to understand the mechanism(s) underlying the *in vitro* and *in vivo* effects of bFGF on lung cancer and endothelial cells and the ability of bFGF inhibitors to suppress the growth of NSCLC *in vitro* and *in vivo.*

Aim 1 Determine the effects of bFGF on *in vitro* growth, survival, motility, invasion and angiogenesis of NSCLC cells and endothelial cells.

Update

Previously, we analyzed the expression of bFGF by western blotting and that of the FGF receptors 1 and 2 by RT-PCR in NSCLC cell lines. During the last year, we expanded these findings to include western blotting analyses of FGF receptors and RT-PCR expression of VEGF and its receptors 1 and 2 (Figure 12). The RT-PCR results (Figure 12A) show that two of the cell lines (1170-I and H441) express components that can mediate both FGF and VEGF signaling in that they expressed the ligands bFGF and VEGF and at last one type of receptor (FGFR and VEGFR). In contrast, the H1975 cell line does not express bFGF but does express the VEGF and both of its receptors. Still, even H1975 has the potential to respond to exogenous bFGF from the microenvironment *in vivo* because these cells express the FGF

receptors 1 and 2. The western blotting data (Figure 12B) show that 1170-I and H441 cells express several molecular forms of the bFGF protein (the lowest is the secreted form 18kDa and the two upper ones are >20 kDa and represent the intracellular higher molecular weight form that are not secreted but can translocate into the cell nucleus. H1975 cells express very low levels of the three species of bFGF proteins. All the cell lines expressed FGFR3, whereas FGFR2 was detected only in H441 cells. Analyses of medium and cell lysates by enzyme-linked immunosorbent assay (ELISA) for VEGF (Figure 12C) revealed that the majority of the factor was secreted into the medium and only a small amount was found in the cell lysates. In contrast, the majority of bFGF was found in the cell lysate and only a small amount was secreted into the medium (Figure 12D). It is noteworthy that there was a good agreement between the RT-PCR, western blotting and ELISA assays. For example almost no bFGF was detected by any of the methods in H1975 cells. In addition, the ELISA assay demonstrated that VEGF is the main secreted angiogenic factor among these three cell lines and that bFGF is primarily intracellular. Thus, VEGF may function as the major angiogenic factor, whereas bFGF may play a role as intracrine mitogenic factor for these cells. Additionally, the FGF receptors may mediate growth stimulation if normal cells in the tumor microenvironment produce bFGF. Of note, these analyses only represent the relative amounts of the factors and receptors not their function and it is important to analyze the function because it is possible that even small amounts of bFGF secreted into the medium will induce cell proliferation, motility and migration.

bFGF Signaling pathway in NSCLC cells. Previously, we reported that treatment of NSCLC cells with bFGF after serum starvation resulted in increased cell proliferation and a rapid and transient increase in phosphorylated ERK (p-p42/44) MAPK and Akt indicating that bFGF was mitogenic and enhanced survival pathway mediated by Akt.

During the last year, we expanded those initial observations to delineate the sequence of molecular events in bFGF signaling in NSCLC and found that: 1) Phosphorylation of FGFR1 was induced within 5 minutes of the addition of 10 ng/ml bFGF to NSCLC 1170-I cells (Figure 13A, top row). This effect was specific for FGFR1 as no increase in FGFR3 was observed after bFGF addition (Figure 13A, row 3). Interestingly, FGFR3 was phosphorylated constitutively, namely even without addition of bFGF. We suggest that endogenous bFGF, perhaps the

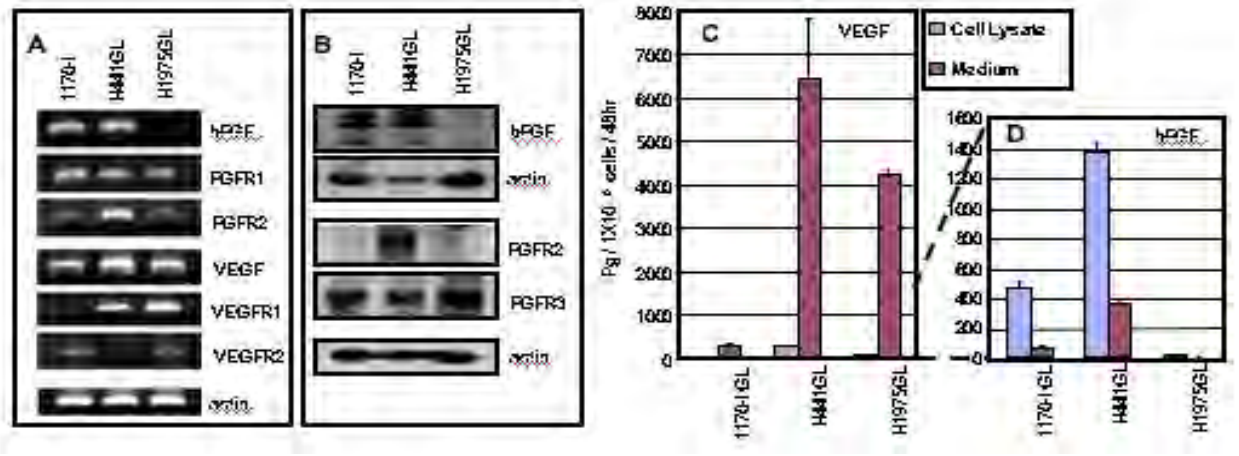


Figure 12. Expression of bFGF and VEGF and their receptors in NSCLC cells. A, B) The NSCLC cell lines indicated above each lane were harvested from nearly confluent cultures and used for extraction of total RNA (A) or total protein (B) and samples were analyzed by RT-PCR and western blotting, respectively for the expression of the factors and receptors indicated to the right of each row. C, D) cells were cultured in serum free medium for 48 hours and then the medium was collected and the cells were lysed and samples of both medium and cell lysate were analyzed using (ELISA) for the level of VEGF (C) or bFGF (D).

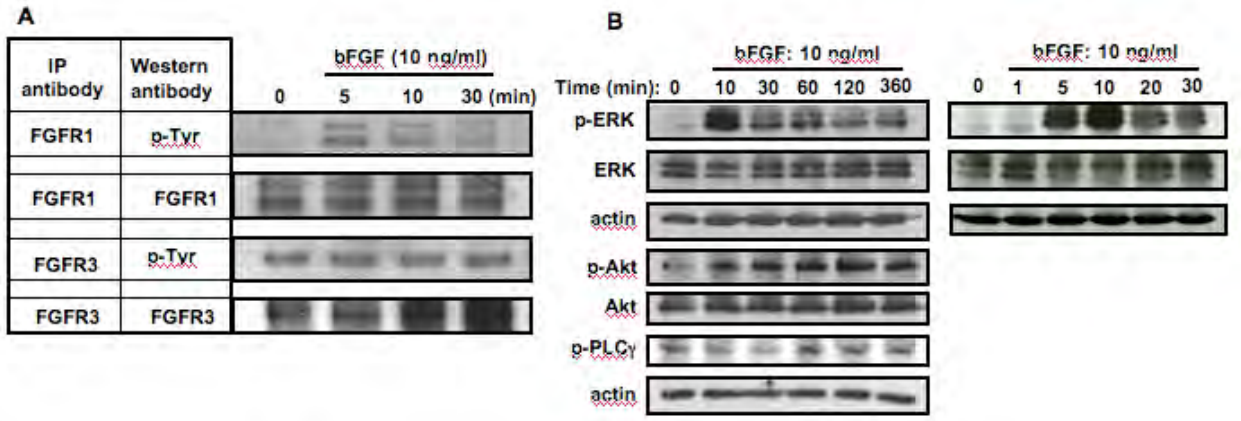
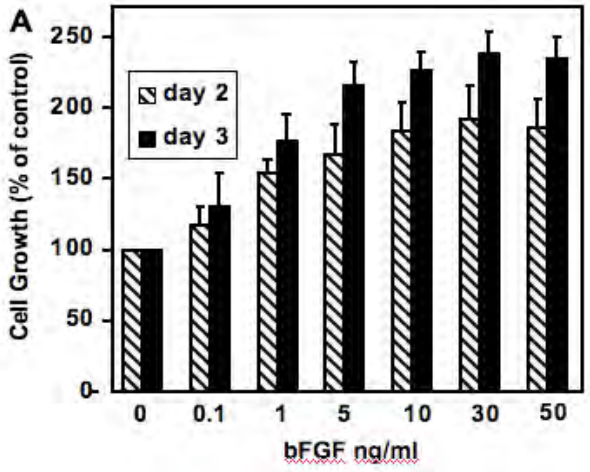


Figure 13. bFGF signaling pathway. 1170-I cells were serum starved for 1 day and then they received bFGF (10 ng/ml), except the control plates for time 0, and different plates were harvested at the times indicated above each lane. A. To assess phosphorylation of FGF receptors, cells were lysed and the total protein fraction was mixed with antibodies against FGFR1 or FGFR3 for immunoprecipitation (IP). The immunoprecipitated proteins were isolated and subjected to gel electrophoresis and blotting. The membranes were then analyzed in a western blotting procedure using antibodies against phosphotyrosine (p-Tyr) or FGFR or FGFR3. B) Cells were treated with bFGF for the indicated times and then the cells were harvested and lysed for extraction of total proteins, which were subjected to western blotting analyses using specific antibodies against the proteins or phosphoproteins indicated to the left of each row.

secreted higher molecular weight variants of bFGF seen in Figure 12B (upper row) activated FGFR3. In other cells, bFGF has been shown to induce autophosphorylation of its receptor and this could lead to one or more of downstream effects by activation of MAPK (ERK1/2), phospholipase C gamma (PLCγ), or PI3K/Akt. We found that in 1170-I NSCLC cells, bFGF activated ERK1/2 and Akt but not the PLCγ pathways. Figure 10B shows that ERK was phosphorylated within 5 min of bFGF addition to the cells. ERK activation of was transient with a peak at 10 min. AKT was also activated by 10 min as indicated by the detection of phosphorylated Akt (Figure 10B, row 4). In contrast, PLCγ was phosphorylated at a low level before bFGF addition and no further or only a minor increase was observed after bFGF addition (Figure 13, row 6). It appears that although FGFR3 appeared to be constitutively activated, this activation did not lead to phosphorylation of either ERK or Akt. However, it is possible that FGFR3 was responsible for the basal phosphorylation of PLCγ.

Effects of bFGF on cell growth. Previously, we have shown that bFGF was mitogenic to several NSCLC cell lines. We have now added data on the 1170-I tumorigenic bronchial epithelial cells. Figure 14 shows that the growth of these cells was stimulated by bFGF in a time- and dose-dependent fashion (Figure 14A) and cell cycle analysis indicated that bFGF decreased the percentage of cells in the G1 phase and increased the percentage of cells in the S phase (DNA synthesis).

Suppression of endogenous bFGF using SiRNA decreases the growth of 1170-I cells. Preliminary results presented in Figure 15 show that treatment of the 1170-I cells with siRNA that targets bFGF causes a decrease in the levels of the endogenous bFGF proteins (B) and this is accompanied by a decreased in cell growth (A)



B

Cell cycle phase	control	bFGF (10 ng/ml)
G1	66.85	▼ 50.17
S	20.75	▲ 36.03
G2	11.9	12.24

Figure 14. A) 1170-I cells were seeded at a density of 2.0 X 10^3 / per well in 96 well plates with medium containing 10% serum and grown for 12 hr. The medium was then replaced with serum-free medium and the cells were incubated for 48 hr. Then the cells received a fresh serum-free medium with the indicated bFGF concentrations or no bFGF (control). After 48 hr and 72 hr, the cell numbers were estimated using the sulforhodamine B (SRB) assay. B) the same cells were cultured in 10-cm diameter dishes with the same medium changes as above and after 72 hr of treatment with bFGF or control medium, the cells were harvested and subjected to cell cycle analysis using flow cytometry.

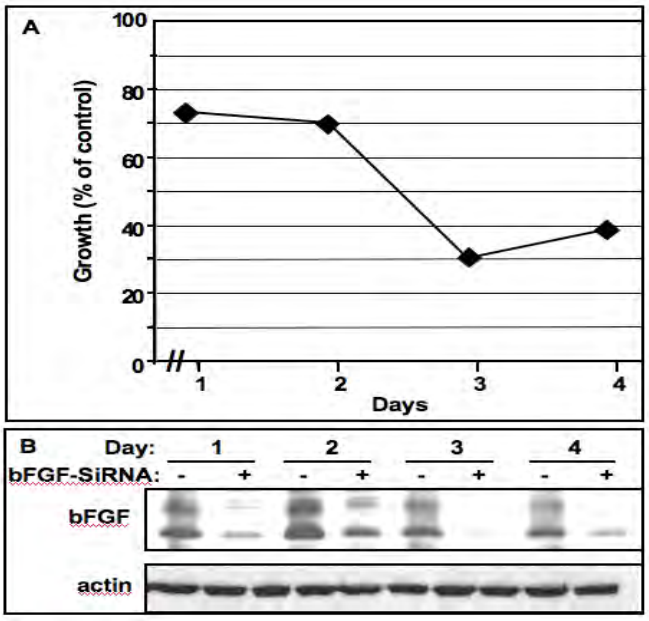


Figure 15. A) 1170-I cells were treated with small interfering RNA (siRNA) targeted against bFGF or control siRNA and the level of bFGF proteins was analyzed by western blotting after 1, 2, 3 and 4 days (B). Cell growth relative to untreated controls was also determined (A).

bFGF enhances the invasion of 1170-I cells. We used a standard invasion assay through a biological extracellular matrix coated on a polyporous membrane in a Boyden chamber to determine whether bFGF exerts invasive properties on the 1170-I cells. Figure 16 shows that when bFGF is placed in the bottom chamber under the coated membrane 1170-I cells are induced to invade through the matrix and through the pores and migrate to the underside of the membrane.

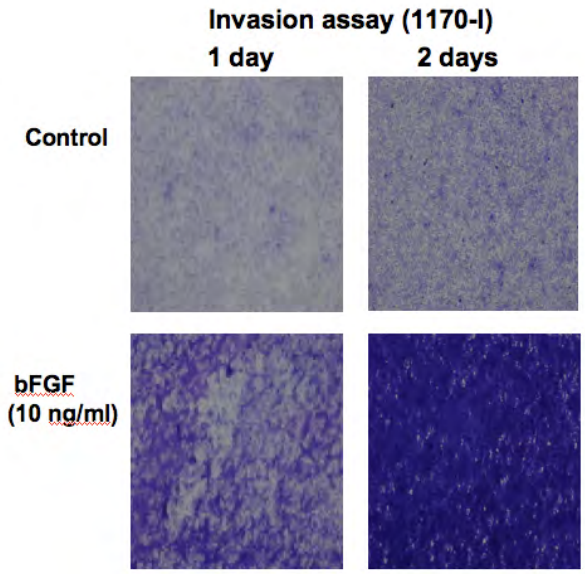


Figure 16. 1170-I cells were placed on top of Matrigel-coated membranes in a Boyden chamber and medium without (control) or with 10 ng/ml bFGF was placed in the bottom chamber. After 24 and 48 hrs, the cells on top of the membrane were removed with a cotton swab and the membranes were stained and the underside was photographed under the microscope. The blue color indicates cells that invaded through the matrix and membrane pores.

Aim 2 Evaluate the relative potency of several inhibitors of bFGF binding to receptor (i.e., TMPP and analogs) in inhibiting effects of bFGF detected in Specific Aim 1 and evaluate the effects of these inhibitors in combination with paclitaxel on *in vitro* growth and survival of tumor cells.

We analyzed the effects of inhibitors of bFGF signaling on the growth of 1170-I cells. The inhibitors included the porphyrin analog, 5,10,15,20-tetrakis(methyl-4-pyridyl)-21*H*,23*H*-porphine-tetra*p*-tosylate salt (TMPP), which can inhibit bFGF and to a lesser extent VEGF binding to their cell surface receptors extracellular domain, the more specific bFGF inhibitor the pyrido-[2,3-d]pyrimidine, PD173074 that binds to the cytoplasmic tyrosine kinase domain of FGF receptor (FGFR1), and Ly294002, the inhibitor of the downstream phosphatidylinositol 3-kinase (PI3K). Figure 17 shows that the three inhibitors suppressed the growth of the 1170-I cells when the cells were cultured for 3 days in the absence of exogenous bFGF in a dose-dependent fashion. The TMPP and PD173074 also suppressed the mitogenic effect of exogenously added bFGF. LY294002 was the most potent inhibitor at the doses used in cells grown with or without bFGF. However, it is not a specific bFGF signaling inhibitor but rather an inhibitor that could also affect signaling by various other growth factors and receptors.

Aim 3 Evaluate anti-tumor activity (growth inhibition, apoptosis, suppression of angiogenesis) of the most effective inhibitor identified in Specific Aim 2 when used alone and in combination with paclitaxel in an orthotopic lung cancer model using luciferase-expressing NSCLC cells for *in vivo* bioluminescence imaging of tumor growth and response to treatment.

Update

We did not work on this aim during the report period.

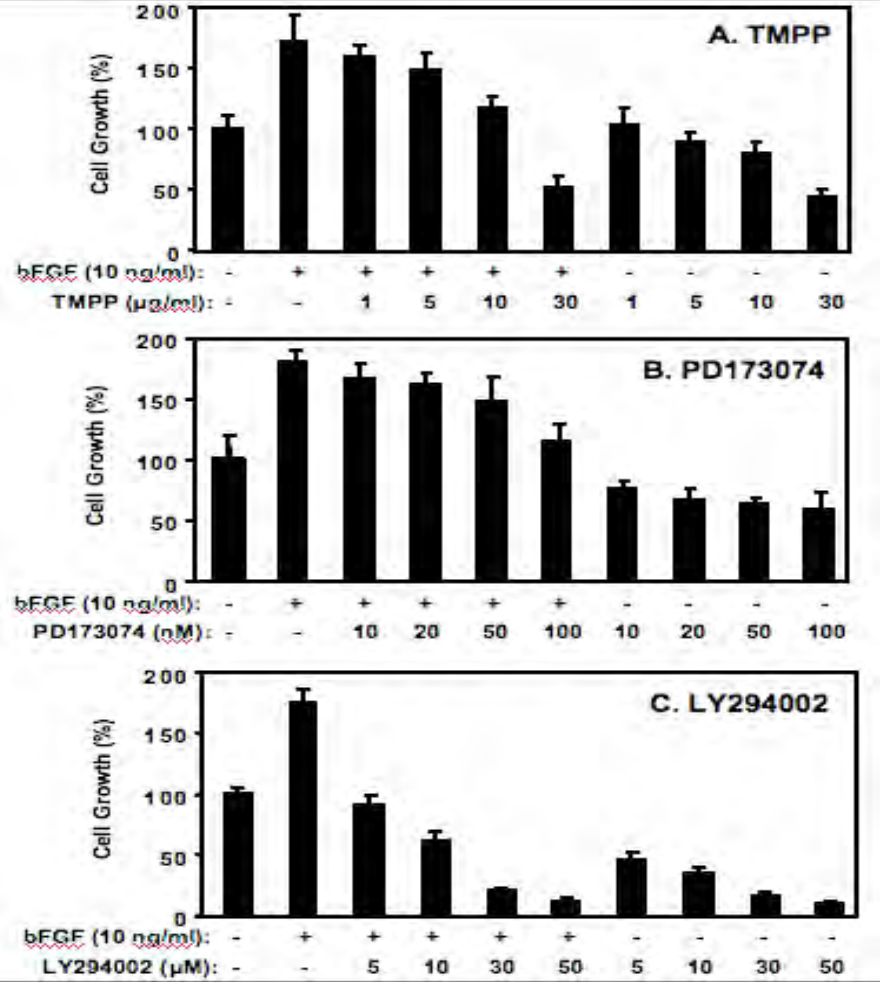


Figure 17. 1170-I cells were seeded at a density of 2.0 X 10^3 / per well in 96 well plates with medium containing 10% serum and grown for 12 hr. The medium was then replaced with serum-free medium and after 48 hrs the cells were treated with 10 ng/ml bFGF or control medium without or with each of the three inhibitors (A, B, and C) at the indicated concentrations. After three additional days, the cells were stained with SRB for estimation of their number. The number of cells in the control cultures grown without bFGF or inhibitors was taken as 100%.

Aim 4 **To investigate the expression of bFGF signaling components (bFGF, FGFR-1, FGFR-2, heparan sulfate, syndecan-1, and FGFR-3) by IHC staining of tissue microarrays (TMAs), and correlate the expression of bFGF/bFGFRs between tumor and non-malignant epithelial cells with angiogenesis.**

Update

During the last year (2007), in collaboration with the Pathology Core (Drs. Carmen Behrens and I.I. Wistuba) and Biostatistics Core (Dr. J. J. Lee), we finalized the analysis of bFGF and its receptors FGFR-1 and -2, and prepared the results for publication. A manuscript describing our findings has been submitted for publication (see Appendix C).

Key research accomplishments

- Discovered that some NSCLC cells express both angiogenesis factors (bFGF and VEGF) and some of their receptors (FGFRs and VEGFRs). Furthermore, bFGF activated its receptor FGFR1 and the downstream pathways including ERK1/2 and Akt but not the PLCγ pathway. Although FGFR3 was constitutively activated it did not activate the same downstream pathways.
- Determined that bFGF acted as a mitogen in 1170-I cells by increasing the entry of cells from the G1 phase of the cell cycle to the S phase.
- Discovered the importance of the bFGF signaling in autocrine stimulation as indicated by the inhibition of cell growth in cells treated with siRNA targeting bFGF. Indirect evidence for this issue has come from the ability of inhibitors like TMPP and PD173074 to suppress the growth of 1170-I cells with and without bFGF stimulation.
- Determined that bFGF induced the migration and invasion of 1170-I cells.
- Discovered bFGF, FGFR1, and FGFR2 are frequently overexpressed in NSCLC in human lung tissue specimens, although different patterns of expression are detected in its two major types. Our findings further suggest that bFGF signaling pathway activation is an early event in the pathogenesis of squamous cell carcinoma of the lung. In addition, the frequent and early overexpression of bFGF and FGFR markers in patients with NSCLC suggests that the activation of the bFGF pathway, which has been proposed to facilitate the development of resistance to anti-angiogenic therapy targeting the vascular endothelial growth factor pathway, is an attractive novel target for lung cancer therapeutic and chemopreventive strategies.

Reportable outcomes

Abstracts

1. Behrens C, Lin H, Lee J, Hong WK, Wistuba II, Lotan R. Differential immunohistochemical expression patterns of fibroblast growth factor-2, receptors 1 and 2, and syndecan-1 in squamous cell carcinoma and adenocarcinoma of the lung. The 98th AACR Annual Meeting, abstract #: 190, 2007.

Manuscripts, submitted

1. Carmen Behrens, Heather Lin, J. Jack Lee, Waun Ki Hong, Ignacio I. Wistuba and Reuben Lotan. Immunohistochemical Expression of Basic Fibroblast Growth Factor and Fibroblast Growth Factor Receptors 1 and 2 in the Pathogenesis of Lung Cancer. Submitted for publication.

Conclusions

We continued our research on bFGF delineating the sequence of molecular events in NSCLC. Our findings this year expand and confirm the data on the mitogenic effect on NSCLC cell lines in a time- and dose-dependent fashion by acting in the transition from the G1 to S phases of the cell cycle. We have also evaluated the bFGF inhibitors TMPP and PD173074 and demonstrated growth suppression in NSCLC cell lines.

Project 5: Targeting mTOR and Ras signaling pathways for lung cancer therapy

(Project Co-leaders: Fadlo R. Khuri, M.D., Shi-Yong Sun, Ph.D.)

Aim 1 To determine whether an mTOR inhibitor inhibits the growth of human NSCLC cells via G1 growth arrest or induction of apoptosis, and to identify the molecular determinants of mTOR inhibitor sensitivity.

This aim was completed and summarized in the previous annual reports.

Aim 2 To determine whether the effect of mTOR inhibitors on the growth of human NSCLC cells is enhanced in the presence of a PI3K inhibitor or a MAPK inhibitor.

This aim has been completed and summarized in the previous reports.

Aim 3 To evaluate the efficacies of the combinations of rapamycin with LY294002 or U0126 in nude mice models of lung cancer xenografts *in vivo*.

Update

We tested the effects of the combination of RAD001 and LY294002 on the growth of lung cancer xenografts in nude mice. In agreement with the results in cell cultures, the combination of RAD001 and LY294002 exhibited a significantly greater effect than RAD001 or LY294002 alone in inhibiting the growth of A549 xenografts ($p < 0.001$) (Figure 18A). During the two-week period of treatment, the tumor sizes in mice receiving both RAD001 and LY294002 were smaller in comparison with other groups receiving either vehicle or single agent treatment (Figure 18A), indicating an effective anticancer efficacy for the combination treatment. In a H460 xenograft model, we began treatments with relatively larger tumors (in average 300-400 nM^3). Both RAD001 and LY294002 alone failed to achieve significant effects on inhibiting the growth of tumors; however, the combination of RAD001 and LY294002 significantly inhibited the growth of H460 xenografts compared to control ($p < 0.05$ or 0.01) (Figure 18B). Collectively, these results clearly demonstrate that co-targeting mTOR and PI3K/Akt signaling exhibits enhanced anticancer efficacy.

We also determined whether continuous RAD001 treatment in cancer xenograft models led to increase in Akt phosphorylation as we observed in cell cultures. By Western blot analysis, we detected p-Akt levels in tumors exposed to RAD001 for 14 days and found that p-Akt levels were significantly increased ($p < 0.05$) in the RAD001-treated group compared to the vehicle control group in both A549 and H460 xenografts (Figure 19A). As expected, p-Akt levels in tumors exposed to the combination of RAD001 and LY294002 were not increased (Figure 19A). Immunohistochemical analysis of p-Akt in H460 xenografts also showed that p-Akt levels was increased in RAD001-treated tumors, but not in tumors exposed to the combination treatment of RAD001 and LY294002 (Figure 19C). Thus, these results clearly indicate that continuous treatment of lung tumors with an mTOR inhibitor in nude mice leads to increase in Akt phosphorylation and this increase can be abrogated by inclusion of a PI3K inhibitor.

Moreover, we determined whether the presence of LY294002 impacted the inhibitory effect of RAD001 on mTORC1 signaling in tumor tissues. As presented in Figure 19B, RAD001 alone

significantly decreased the levels of p-S6 ($p < 0.001$), indicating that RAD001 indeed inhibits mTORC1 signaling; however, the presence of LY294002 further reduced the levels of p-S6, which were significantly lower than those in tumors exposed to RAD001 alone ($p < 0.05$ or 0.01). Thus, these results indicate that co-treatment of tumors with an mTOR inhibitor (e.g., RAD001) and a PI3K inhibitor (e.g., LY294002) not only blocks RAD001-induced Akt phosphorylation, but also exhibits an enhanced effect on inhibiting mTORC1 signaling.

Aim 4 To conduct a pilot clinical biochemical induction trial to investigate the effect of RAD001 in operable NSCLC patients and identify molecular determinants of RAD001 sensitivity and prognosis.

Update

We have launched this Phase Ib protocol effective February, 2007. After screening 7 eligible patients, with 6 of 7 declining due to the randomization to placebo, we successfully amended the study to remove the placebo arm, obtaining approval from the FDA and Department of defense Human Protection Committees as well as Emory's IRB, and have now accrued 3 total patients on this trial. One of these 3 patients had drug discontinuation, due to Grade 3 nausea and vomiting, after 8 days. The other 2 completed 21 days of RAD001 pre-operatively, as per protocol, and in fact had disease stabilization with minor reduction in SUV on PET scans. Accrual continues on this study.

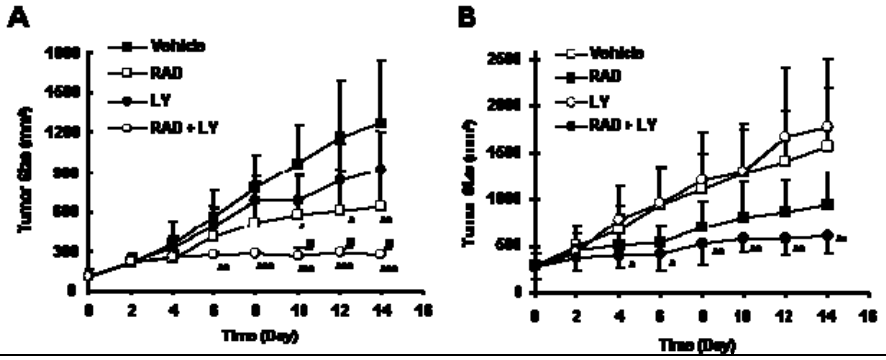


Figure 18. **Combination of RAD001 and LY294002 augments growth inhibition of lung cancer xenografts in nude mice (A and B).** Four groups mice with either A549 (*A*) or H460 (*B*) xenografts were treated with vehicle, RAD001 (RAD) alone, LY294002 (LY) alone and RAD001 plus LY294002 on the same day after grouping. After 14 days, tumor sizes were measured once every two days. Each measurement is a mean ± SD (n=6). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to vehicle control; # $p < 0.05$ compared to RAD001 treatment.

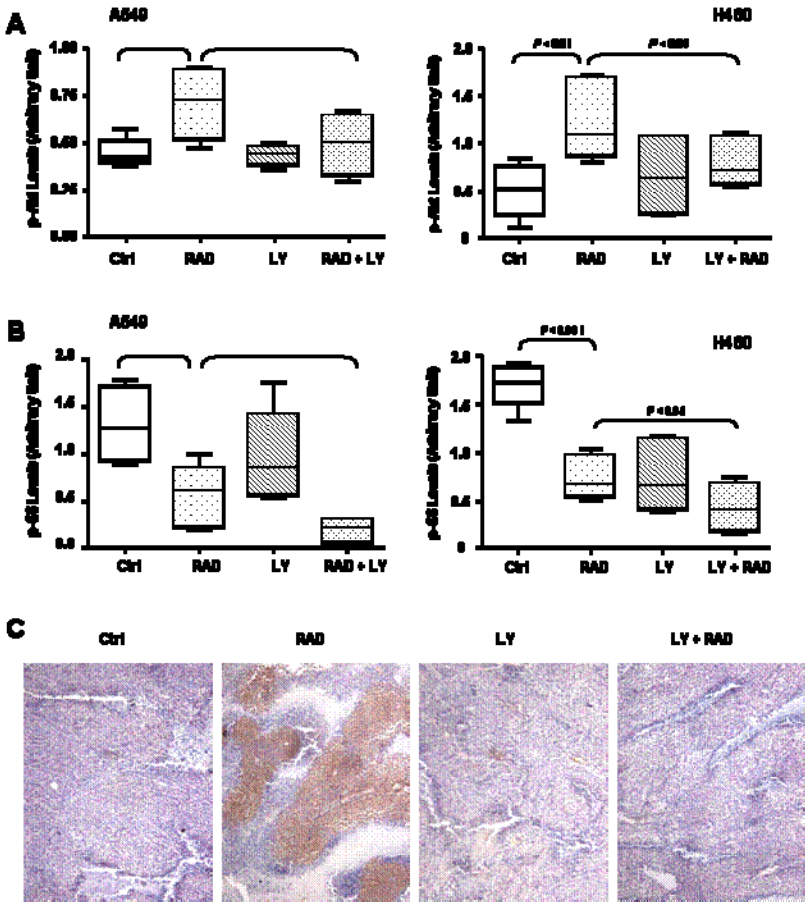


Figure 19. Detection of p-Akt and p-S6 levels in tumor tissues. *A* and *B*, Tissue from each tumor was homogenized for preparation of whole-cell protein lysates and subsequent analyses of p-Akt (*A*) and p-S6 (*B*) by Western blot analysis. The results were quantitated using Kodak Image Station 2000R and presented as a mean ± SD (n=6). *C*, p-Akt in H460 xenografts was stained with IHC.

Key Research Accomplishments

- Co-targeting of mTOR and PI3K/Akt signaling exerted enhanced anticancer activity in lung cancer xenograft models.
- Co-targeting of mTOR and PI3K/Akt signaling enhances inhibition of mTOR signaling while preventing Akt activation by inhibition of mTOR signaling.

Reportable Outcomes

Manuscripts published in peer-reviewed Journals

1. Wang X, Yue P, Chan C-B, Ye K, Ueda T, Watanabe-Fukunaga R, Fukunaga R, Fu H, Khuri F, Sun S-Y. Inhibition of mammalian target of rapamycin induces phosphotidylinositol 3-kinase-dependent and Mnk-mediated eukaryotic translation initiation factor 4E phosphorylation. Mol Cell Biol 27(21)7405-13, 2007.

Manuscript, in preparation

1. Wang X, Yue P, Young Ae Kim, Fu H, Khuri, Sun S-Y. Inhibition of mTOR/raptor complex initiates phosphatidylinositol 3 kinase-dependent Akt activation which counteracts mTOR inhibitors' anticancer efficacy.

Conclusions

Targeting the mTOR axis appears to be a promising strategy against lung cancer. Given the nature of the complexity of lung cancer signaling pathways, including mTOR signaling, it is essential to understand the biology of lung cancer and the mechanism of action for the therapeutics of interest in order to efficiently treat lung cancer through application of mechanism-driven therapeutic regimens. Thus, we have demonstrated the scientific rationale for our effort in pursuing mTOR-targeted lung cancer therapy.

Project 6: Identification and Evaluation of Molecular Markers in Non-Small Cell Lung Cancer (NSCLC)

(PI and co-PI: Ralf Krahe, Ph.D., Li Mao, M.D)

A better understanding of the lung cancer biology and an identification of genes involved in tumor initiation, progression and metastasis are an important first step leading to the development of new prognostic markers and targets for therapy. In the same context, identification of reliable predictive markers for response or resistance to therapy in NSCLC patients is also desperately desired for optimal delivery of targeted therapy and/or standard chemotherapy. The proposed studies aim to identify the two types of markers that would eventually help develop smarter clinical trials, which will selectively recruit patients who are more likely to respond to one regimen over another and lead to improvement of overall therapeutic outcomes.

Aim 1 To expression profile by DNA microarray technology aerodigestive cancers - with primary focus on adenocarcinoma and squamous cell carcinoma (SCC) of the lung, and head and neck squamous cell carcinoma (HNSCC), including primary tumors and normal adjacent tissue, and (where available) metastatic lesions.

Update

Expression profiling of HNSCC and NSCLC. A manuscript, the results of which were presented in last year's update, reporting our findings on expression profiling of HNSCC samples has been provisionally accepted by *Head & Neck* (Colella S, Richards KL, Bachinski LL, Baggerly KA, Tsavachidis S, Lang JC, Schuller DE, and Krahe R. Molecular Signatures of Metastasis in Head and Neck Cancer (provisionally accepted, *Head Neck*). Briefly, using HNSCC as a prototypic solid tumor of epithelial origin, we identified genes involved in head and neck cancer initiation, progression and metastasis. Taking advantage of genetically matched samples, including normal adjacent tissues (NAT), primary tumors (PT) and lymph node metastases (LNM), we identified tumor-associated gene expression signatures that were common to both primary tumors and metastases. These results are consistent with the notion that the metastatic potential is already encoded in the bulk of the primary tumor.

Our study derived a metastatic signature from a direct comparison between PT and genetically matched LNM. Although our metastatic signature was derived from and is specific to metastases, it shares genes in common, and even more strikingly, shares a similar set of biologic pathways and processes with previously published HNSCC metastatic signatures derived from primary tumors based on clinical endpoint data. This implies that while metastasis requires changes in expression of genes in many pathways/processes, the order and timing of these changes is less important, since our signature, derived from expression changes specific

to metastases, matches other signatures derived from primary tumors. Through the incorporation of genetically matching tissue samples (NAT, PT and LNM), our experimental design enabled us to objectively address the unresolved issue of genetic heterogeneity between tumor and metastasis tissues from different patients.

To date, we have completed expression profiling on 8 matched tumor/normal adjacent tissue (T/N) pairs of lung cancers, 5 adenocarcinomas and 3 squamous cell carcinomas (SCC), from an initial total of 22 NSCLC T/N pairs. Additional samples are being collected by the Pathology Core (LAB03-0320). At this stage, we aim to have 25-30 NSCLC normal/tumor pairs profiled over the coming year. Based on the limited expression profiling data for NSCLC currently in hand, it is impossible to draw statistically meaningful conclusions for the lung cancer part of the study, or to perform meaningful comparisons with the HNSCC part of the study.

Aim 2 To DNA profile the same samples by complementing DNA approaches to stratify RNA expression profiles on the basis of their corresponding DNA profiles.

Update

DNA profiling of corresponding HNSCC and NSCLC tumors. Analogous to our NSCLC study design, we performed genomic profiling of the same HNSCC samples on which we performed transcriptomic profiling (see above Aim 1) using a second generation SNPChip array (Affymetrix 10K *Xba*I array). Using 24 genetically matched NAT and PT pairs and 12 matching MLN, we genotyped a total of 8,430 tag SNPs. Analysis of the genotype data with GeneSpring GTv2 (Agilent) for loss-of-heterozygosity (LOH) and autozygosity, indicating allelic imbalance (AI), showed that the majority of AI events occurs from normal to primary tumor, while the transition from primary tumor to metastasis was marked by very little additional events (0-2%). In the NAT/PT comparison, we identified five regions with significant AI: 9p24.1-p23 (38%), 5q33.3 (25%), 3q25.32-q26.1, 13q14 and 17p13 (all 21%), and 3p23-p22.3 (13%) (Figure 20A). These regions contain several excellent candidate tumor suppressor genes (TSG) with known functions in cell growth, differentiation, cell cycle regulation and oncogenic transformation, some of which have been implicated in other tumors: for example, *PTPRD* (9p24.1-p23), *EBF1* and *IL12B* (5q33.3), *RARES*, *IL12A* and *SMC4* (3q25), *DNAH9* and *MAP2K4* (17p13.1-p12). In the PT/LNM comparison, we identified several regions of AI, of which 11q23 stood out as the most common event (25%) (Figure 20B). Among a total of about 60 genes, this region of AI, likely amplification, contains two genes (*TAGLN* and *DDX6*) involved in transformation and proliferation.

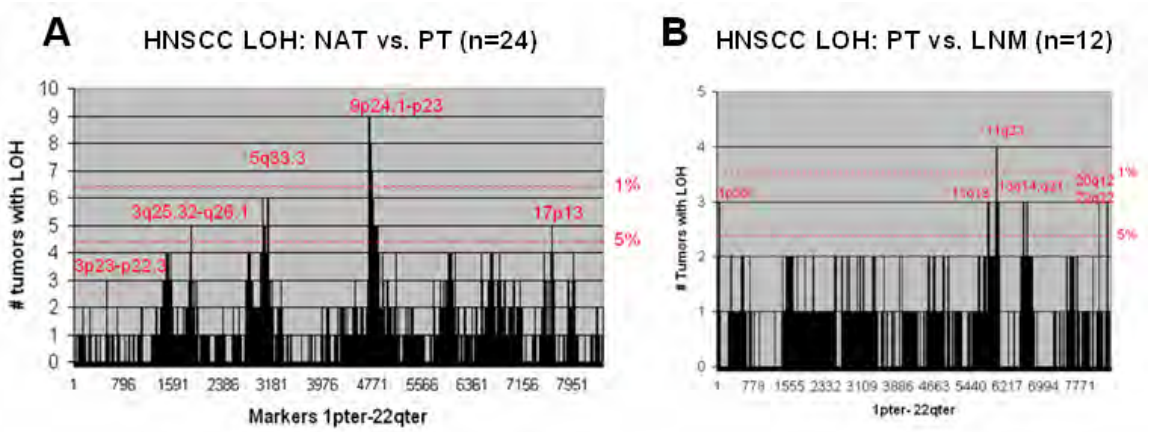


Figure 20. LOH analysis of HNSCC samples. The 1% and 5% lines indicate the percentage of markers for all markers interrogated (n=8,430) showing LOH.

It is worth noting that in a recent large-scale high-density scan on 371 primary adenocarcinomas with a SNPChip array interrogating ~250,000 SNPs, several of the same regions (9p, 5q, 13q and 17p) were identified as regions of recurrent loss/gain, both large-scale and focal deletions/amplifications (Weir et al., 2007). Moreover, in ~6% of cases *PTPRD* had somatic mutations.

Interestingly, both *PTPRD* and *RARRES1* have CpG islands in their 5' promoter regions and may therefore be subject to epigenetic inactivation by promoter hypermethylation (Aim 3). As hypermethylation is a "hit" that is functionally equivalent to loss, we will also investigate both of these genes in Aim 3.

To date, we have extracted DNA from 21 NSCLCs (see above). Originally, we proposed to correlate their DNA and RNA profiles. Because of the issues related to sample procurement, it may not be feasible to obtain the necessary number of matching RNA/DNA samples to obtain statistical power. We, therefore, obtained DNA samples without matching RNA samples from 108 NSCLC patients with complete clinical and follow-up data (56 adenocarcinomas, 48 SCC, and 25 other lung cancers) for a total of 129 PT and 45 matching NAT/PT pairs, which we will use for genomic profiling if suitable samples with matching RNA/DNA cannot be obtained. As SNP microarray technologies and the bioinformatics tools for their analysis have continued to evolve over the last year, we can now analyze our data for DNA copy number and LOH using the same data set. We have extensive experience with these tools now; therefore, the completion of this aim should be less time and labor intensive.

Aim 3 To evaluate the contribution of promoter hypermethylation and transcriptional inactivation of known cancer genes subject to epigenetic silencing to cancer phenotype.

Update

Methylation profiling of NSCLC tumors. Last year, we reported on the methylation profiling of 9 genes known (or suspected) to be methylated in head and neck and lung cancer (*APC*, *MLH1*, *RASSF1*, *MGMT*, *CDKN2A*/*p14* and *p16*, *ATM*, *GSTP1* and *TCF21*) on 24 pairs of matched HNSCC primary tumors and normal adjacent tissue, plus an additional matched 14 metastatic lymph nodes, and 21 NSCLCs and their matched normal samples.

Of the genes we assayed, the transcription factor *TCF21* was the most often hypermethylated (≥30% methylation) gene in both HNSCC (96%, n=24) and NSCLC (95%, n=21). To confirm our initial finding, we obtained additional DNA samples from 108 NSCLC patients with complete clinical and follow-up data (56 adenocarcinomas, 48 SCC, and 25 other lung cancers) for a total of 129 PT and 45 matching NAT/PT pairs. Overall, 73% of lung cancers showed *TCF21* hypermethylation (n=129). Adenocarcinomas and SCCs together showed hypermethylation in 78% (n=100) of tumors, while other lung cancers showed hypermethylation in 64% of cases (n=25). Adenocarcinomas compared to SCCs showed slightly higher levels of (40%±2.5 vs. 37%±2.7) and more frequent (90% vs. 70%) methylation, respectively.

To determine the effect of *TCF21* promoter hypermethylation on gene expression, we together with Dr. Ignacio Wistuba (Pathology Core) performed TCF21 protein immunohistochemical (IHC) analysis on a TMA of 300 lung cancer cases (Figure 21). Consistent with our DNA-based methylation data 253 of 300 cases [85%; 166/191 (87%) adenocarcinoma and 87/109 (80%) squamous cell carcinoma) showed no or significantly decreased TCF21 expression. Correlation of methylation status with available clinical factors (Biostatistics Core), showed a significant correlation with histology (adenocarcinoma, p = 0.0026), sex (females, p = 0.0209), avnd smoking status (never smokers, p = 0.0476). However, race, TNM stage, and prognosis were

not significantly associated. We are in the process of performing multivariate analysis to determine if these factors are independently associated. Given the interesting association of histology, sex, and smoking-status (adenocarcinoma in female never-smokers), which is reminiscent of recent findings for *EGFR* mutations in lung cancer patients, we investigated the possibility that EGFR mutation status might predict TCF21 expression. Multivariate analysis showed that histology, but not gender or smoking status was associated with low TCF21 expression. However, when adenocarcinoma cases (n=172) were considered alone by both univariate and multivariate analysis, no significant correlation was observed.

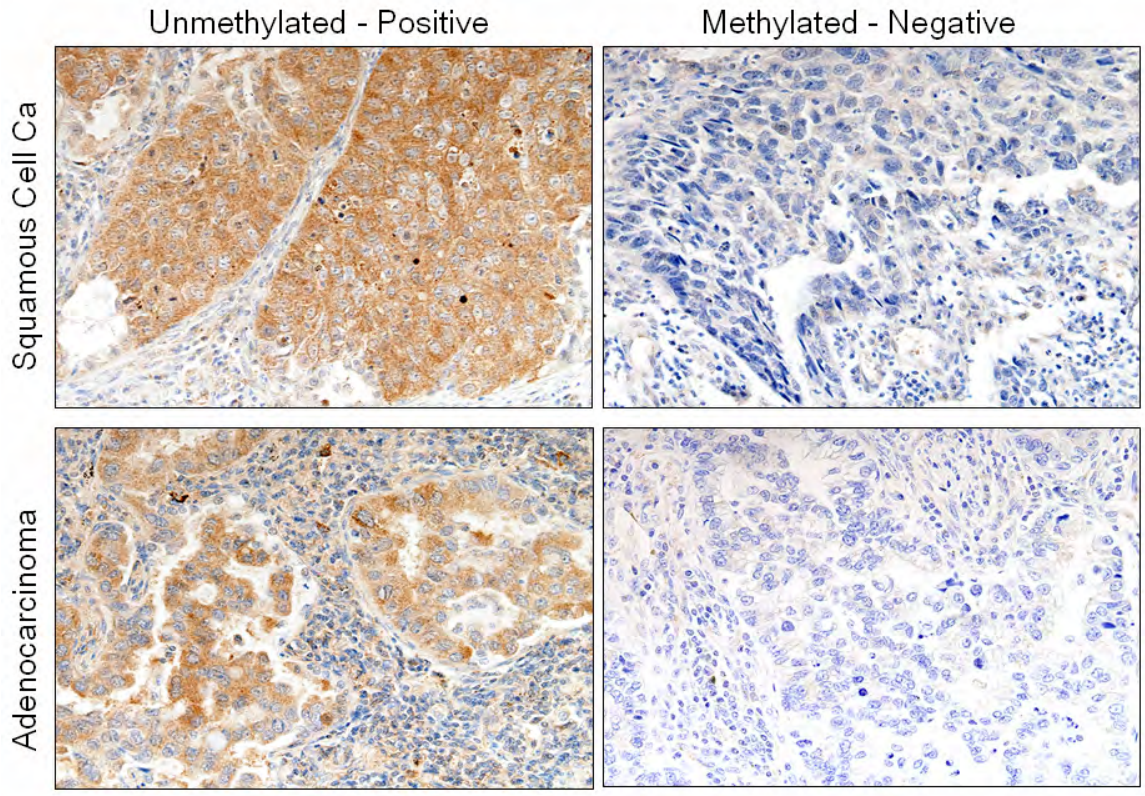


Figure 21. IHC for TCF21 expression on a lung cancer tissue microarray. 253 of 300 lung cancer cases (85%) showed no or significantly decreased TCF21 expression. Methylation status correlated with level of protein expression. To determine the expression and pattern of TCF21 protein expression in NSCLCs, we performed IHC for TCF21 using antibodies against TCF21 protein (sc-15007, Santa Cruz Biotechnology, Santa Cruz, CA). Archival FFPE tissues from surgically resected lung cancer specimens containing tumor and adjacent normal tissues were obtained from 281 NSCLCs (172 adenocarcinomas and 109 squamous cell carcinomas). Tumors have been histologically examined and classified using the 2004 World Health Organization (WHO) classification. All cases have been placed in triplicate 1-mm diameter tissue cores per tumor specimen obtained from central, intermediate, and peripheral tumor areas. Detailed clinical and pathological information, including demographic, smoking history (never- and ever-smokers) and status (never, former, and current smokers), clinical and pathologic TNM staging, overall survival and time of recurrence, are available for all cases.

TCF21 is an important basic helix-loop-helix transcription factor that plays an important role in the epithelial-mesenchymal transition (EMT). Because TWIST1 is another important family member of this group of factors with an established role as an oncogene in EMT, we are currently also evaluating the same cases for TWIST1 expression by IHC. We conclude that based on our methylation profiling, *TCF21* promoter hypermethylation and TCF21 expression are good biomarker of both early lung and head and neck cancer (manuscript in preparation).

Over the last year, we added a novel tumor suppressor gene, *CHD5*, that is involved in the transcriptional regulation of *p53* (another important tumor suppressor gene that is often mutated

in lung cancer) to our panel of methylatable candidate genes. We have shown that the *CHD5* promoter is subject to hypermethylation of varying levels in several tumor types, including lung cancer and head and HNSCC (~20% of samples). We are currently following up this initial observation in a larger panel of lung cancer samples. We have tried-and-proven methylation assays for another 10 genes in hand and are currently testing four additional assays for other candidate genes [*PTPRD* and *RARRES1* (see Aim 2), and *DCLRE1A* and *DCLRE1B*], all of which we plan to evaluate over the next year.

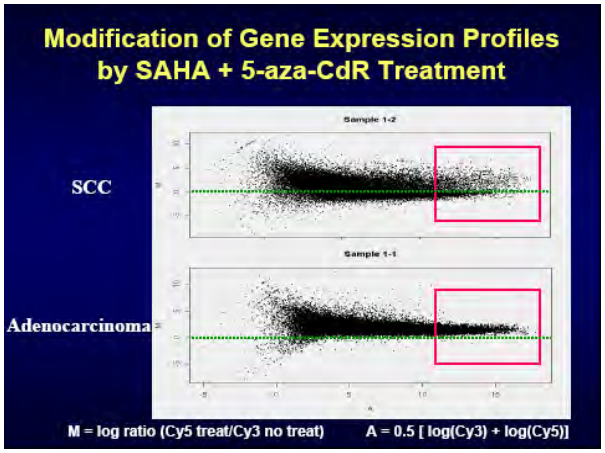


Figure 22. Gene expression in different tumors treated with SAHA and 5AZ.

Aim 4 To determine protein signatures of treatments of erlotinib and other therapeutic agents, alone or in combination, in NSCLC and identify molecular predictors of response.

Update

Biologic properties of cells are virtually determined by their cellular protein levels and structural modifications. Cancers in the upper aerodigestive tract carry a number of abnormally expressed proteins and posttranslational modifications, which are important in cancer progression. Additionally, key signaling pathways critical in cell survival and response to therapeutic agents, particularly molecular targeted agents, are influenced by inherited genetic background of individual patients. These differences, both developed in tumorigenesis and inherited from their parents, determine potential cellular responses to agents to be used for killing or inhibiting tumor cells.

In the last report, we reported that we have successfully established 7 models from 7 individual patients. In this reporting period, we established an additional 7 models from 7 different patients. The general characteristics of these 14 heterotransplant lung cancer models are shown below in Table 1.

We have tested two tumor models, one adenocarcinoma and one squamous cell carcinoma for their response to SAHA (HDAC inhibitor) and 5-Aza-citidine (DNMT inhibitor) treatment. We expected the treatment would activate expression of a panel of genes which were silenced in tumorigenesis due to promoter DNA methylation or histone modifications because the combination of SAHA and 5-AZ would result in DNA demethylation and histone acetylation. After one of week treatment, we observed that the squamous cell tumor model responded to the treatment and showed significant up-regulation of gene expression in a panel of genes (Figure 22, upper panel) whereas the adenocarcina did not show any detectable significant response to the treatment in terms of gene expression regulation (Figure 22, lower panel). The results suggest that different lung cancers may respond differently to the treatment regimen, which may be determined by the underlying biology of the tumors. This observation also suggests the possibility of using these tumor models to develop biomarkers able to predict which tumors would likely benefit from the treatment regimen.

Table 1. Characteristics of heterotransplant lung cancer models.

DIAGNOSIS	DIFFERENTIATION	GENDER	AGE	ETHNICITY	Stage
Adenocarcinoma	Moderately	Male	74	Caucasian	IA
Squamous Cell Ca.	Moderately	Male	65	Caucasian	IIA
NSCLC	Poorly	Female	61	Caucasian	IB
Squamous Cell Ca.	Moderately	Male	53	Caucasian	IIB
Adenocarcinoma	Moderately	Female	51	Black	IIB
Adenocarcinoma	Poorly	Male	71	Caucasian	IA
Squamous Cell Ca.	Moderately	Female	64	Caucasian	IIA
Adenocarcinoma	Well	Male	58	Caucasian	IB
Adenocarcinoma	Well	Male	72	Caucasian	IB
Adenocarcinoma	Moderately	Female	64	Caucasian	IA
Squamous Cell Ca.	Poorly	Female	63	Caucasian	IB
NSCLC	Poorly	Female	79	Caucasian	IIB
Adenocarcinoma	Poorly	Male	54	Caucasian	IB
Adenocarcinoma	Poorly	Male	83	Caucasian	IIB

Aim 5 To determine a clinical utility of the molecular predictors.

Update

Any biomarker developed must be tested in clinical trials to determine its sensitivity and specificity and to validate its utility in the clinic. Additionally, the assay should be tested for its ability to use small amounts of tissues or body fluids obtainable in clinical practice. The most sensitive and reliable clinical tests currently available are antibody-based assays.

Overexpression of EGFR is common in head and neck squamous cell carcinoma (HNSCC). To correlate with research by my Co-PI, Dr. Krahe, in Aims 1-3, we analyzed the genetic nature of *EGFR* gene including mutations and gene copy numbers in HNSCC and its clinical correlations in 134 HNSCC tumors. Aberrant *EGFR* copy numbers were found in 32 (24%) of 134 tumors, including 22 (17%) with increased copy number and 10 (7%) with decreased copy number. Patients whose tumors had *EGFR* copy number alterations (particularly patients with increased copy numbers) had significantly poorer overall, cancer-specific, and disease-free survivals compared with patients with normal copy numbers ($p < 0.0001$). At 5 years after initial diagnosis, 20 of the 22 (91%) patients with increased copy number died of disease compared with 30 of the 102 (29%) patients with normal copy number. No mutations on *EGFR* exons 18, 19, and 21 were detected in any of the tumors. We conclude that a subset of HNSCC manifests *EGFR* copy number alterations, and this is associated with a poor clinical outcome, suggesting a biologic role of the alterations. (Temam, J Clin Oncol 25:2164-2170, 2007)

In another study, we analyzed potential biologic roles of ΔDNMT3B, a newly identified DNMT3B subfamily in promoter-specific DNA methylation regulation in human lung cancer. We have previously shown a strong correlation between the promoter methylation of *RASSF1A* gene but not *p16* gene and expression of *ΔDNMT3B4* in primary lung cancer, suggesting a role of ΔDNMT3B in regulating promoter-specific methylation of common tumor suppressor genes in tumorigenesis. We now provide the first experimental evidence showing a direct involvement of ΔDNMT3B4 in regulating *RASSF1A* promoter methylation in human lung cancer cells.

Knockdown of Δ*DNMT3B4* expression by small interfering RNA resulted in a rapid demethylation of *RASSF1A* promoter and reexpression of *RASSF1A* mRNA but had no effect on p16 promoter in the lung cancer cells. Conversely, normal bronchial epithelial cells with stably transfected Δ*DNMT3B4* gained an increased DNA methylation in *RASSF1A* promoter but not *p16* promoter. We conclude that promoter DNA methylation can be differentially regulated and Δ DNMT3Bs are involved in regulation of such promoter-specific *de novo* DNA methylation. (Wang et al, Cancer Res 67:10647–52, 2007)

Key Research Accomplishments

- DNA profiling of 28 HNSCC matched T/N pairs and 14 matching lymph node metastases identified regions of LOH and allelic imbalance in HNSCC that are shared with adenocarcinomas.
- Genotyping of 8,430 tag SNPs from 24 genetically matched NAT and PT pairs and 12 matching lymph node metastases.
- Completed gene-specific hypermethylation analysis of 10 genes in HNSCC and NSCLC samples.
- Confirmed *TCF21* promoter hypermethylation and TCF21 expression as good biomarker of both early lung and head and neck cancer.
- Established 14 heterotransplant primary NSCLC tumor models, which will allow us to evaluate target therapeutic agents and to initiate biomarker discovery experiments.
- Identified ΔDNMT3B4 as regulator of RASSF1A promoter in human lung cancer cells.

Reportable Outcomes

Resources

Additional 7 heterotransplant primary NSCLC tumor models have been established which allowed us to initiate evaluation of target therapeutic agents and biomarker discovery experiments. Three peer-reviewed publications were partially supported by this project.

Manuscripts published in peer-reviewed Journals

1. Colella S, Richards KL, Bachinski LL, Baggerly KA, Tsavachidis S, Lang JC, Schuller DE, and Krahe R. Molecular Signatures of Metastasis in Head and Neck Cancer (provisionally accepted, *Head Neck*).
2. Temam S, Kawaguchi H, El-Naggar AK, Jelinek J, Tang H, Liu D, Lang W, Issa JP, Lee JJ, Mao L. Epidermal growth factor receptor copy number alterations correlate with poor clinical outcome in patients with head and neck squamous cancer. J Clin Oncol 25(16):2152-5, 2007.
3. Wang J, Bhutan M, Pathak AK, Lang W, Ren H, Jelinek J, He R, Shen L, Issa JP, and Mao L. *ΔDNMT3B* variants regulate DNA methylation in a promoter-specific manner. Cancer Res, 67(22): 10647-10652, 2007.

Conclusions

We will continue to perform RNA and DNA profiling on available samples to identify genes and genomic regions that are altered in NSCLC. We will interrogate additional candidate methylatable genes as potential tumor suppressor genes in NSCLC, similar to the way we have approached TCF21, to identify their potential as biomarkers.

We will continue to establish additional heterotransplant tumor models as planned. We will start to characterize molecular signatures of the tumor models such as gene expression profiles and mutations of key tumor suppressor genes and oncogenes in both primary tumors and the corresponding heterotransplant tumors. The information will be important for development of biomarkers for potential personalized treatment experimentations. We will expand our treatment to more tumor models and analyze responses with molecular signatures to identify potential biomarkers. The models will also be used to test other novel agents through expanded program supported by other funding sources to further enhance the impact of the models in oncology preclinical studies.

Core B: Biostatistics & Data Management Core

(Core Director: J. Jack Lee, Ph.D.)

The Biostatistics and Data Management Core has continued to work with all IMPACT Projects in their research efforts, especially in the area of biostatistical support in clinical trial design, implementation, and analysis of experimental results. We also developed statistical methods to enhance the design and analysis pertinent to the lung cancer research.

Specific Aims:

1. To ensure that the results of all projects are based on well-designed experiments and are appropriately interpreted by providing experimental design; sample size estimates; power calculations; and integrated, comprehensive analysis for each basic science, pre-clinical, and clinical study.
2. To develop a data management system that integrates clinical, pathological, and basic science data while providing data integrity through process tracking and quality control.
3. To provide statistical and data management support for genomic and imaging studies including microarray, proteomics, and molecular targeted imaging.
4. To develop and adapt innovative statistical methods pertinent to biomarker-integrated translational lung cancer studies.
5. To produce statistical reports for all projects.
6. To collaborate and assist all project investigators with the publication of scientific results.

Update

One major effort in the third year of funding was to continue providing statistical support in the design and revision of the clinical trial proposed in Project 1. We have continued to assist the Project PI in the revisions of the trial: “A Phase II Study of Tarceva (erlotinib) in Combination with Chemoradiation in Patients with Stage III A/B Non-Small Cell Lung Cancer” (PI: Dr. Ritsuko R. Komaki). The study is now activated at M. D. Anderson.

In addition, we have provided statistical support for the design and implementation of a clinical trial proposed in the Developmental Research Project 1 (see DRP-1 below) named: “Treatment of Malignant Pleural Effusion with ZD6474 a Novel Vascular Endothelial Growth Factor Receptor (VEGFR) and Epidermal Growth Factor Receptor (EGFR) Tyrosine Kinase Inhibitor” (PI: Dr. Roy Herbst). This study is now activated at M. D. Anderson as well.

We have worked with Drs. Mao (Project 6) and Wistuba in the analysis of methylation data from lung cancer cases. In addition, we worked with Dr. Wistuba on Projects 2, 3, and 4 regarding the TMA studies on a battery of markers. Specifically, we have worked with Dr. Wistuba on the statistical analyses on expression of the multiple biomarkers (e.g., EGFR and KRAS) with 306 patients. We also worked on the TCF21/EGFR analyses with 202 patients. We continue to work on the study with GRP78, IL-11R and EphA5 on 301 NSCLC patients with Dr. Masanori Sato (Project 3). Abstracts of the results for the latter two projects were submitted to the 2007 AACR meeting (Sun et al., 2007; Massarelli et al., 2007; Behren et al., 2007). We have also continued working with Dr. Gelovani, Project 2, in evaluation of the statistical aspects for the proposed clinical trial using novel imaging techniques.

Key Research Accomplishments

- Continued to provide statistical support in the clinical trial design and revision for Project 1 and DRP-1.
- Provided data analysis for Projects 2, 3, 6, and Pathology Core.
- Continued to work closely with the Project 4 PI (Dr. Reuben Lotan) on synergy studies of combination drug treatment in cell lines to determine whether the effect is synergistic, additive, or antagonistic.
- Generalized and refined available methods to allow flexible modeling of drug interaction to account for the possibility that the combination may produce synergistic effect in certain dose ranges but additive or antagonistic in other dose ranges. The magnitude of drug interaction can also vary from dose to dose.
- Developed the code for implementing two new statistical methods – one parametric generalized response surface model and one semi-parametric model, which allow more general interaction patterns for the drug interaction and relax the restrictions of the existing methods.
- Developed methods to construct the confidence interval for the interaction index for the Emax model.
- Developed an additive hazards model with time-varying coefficients.
- Developed a new Bayesian cure rate model to estimate the cure rate and threshold.
- Developed a cure rate model with covariate measurement errors.
- Developed a dose-finding trial design with multiple drugs.

All these statistical methodology publications acknowledged the support of the DoD grant IMPACT and are found in Appendix C.

Reportable Outcomes

Manuscripts published in peer-reviewed

1. Li, H., Yin, G. and Zhou, Y. Local likelihood with time-varying coefficient additive hazards model. The Canadian Journal of Statistics 35, 321-337, 2007.
2. Lee JJ, Kong M, Ayers GD, Lotan R. Interaction index and different methods for determining drug interaction in combination therapy. J of Biopharm Stat 17:461-80, 2007.
3. Kong M, Lee JJ. A semiparametric model for assessing drug interaction. Biometrics, Sept 27, 2007, epub ahead of publication.

Manuscripts in press, review, revision, or submission

1. Lee JJ, Kong M. Confidence Interval of Interaction Index for Assessing Multiple Drug Interaction. Statistics in Biopharmaceutical Research, In press, 2008.

2. Nieto-Barajas, L. E. and Yin, G. Bayesian semiparametric cure rate model with an unknown threshold. Scandinavian Journal of Statistics, In press, 2008.
3. Yin, G. and Yuan, Y. Bayesian dose-finding for drug combinations by copula regression. In revision for Applied Statistics, 2008.
4. Ma, Y. and Yin, G. Cure rate model with mismeasured covariates under transformation. In revision for Journal of the American Statistical Association, 2008.
5. Lee JJ, Lin HY, Liu DD, and Kong M. Applying Emax model and interaction index for assessing drug interaction in combination studies. Submitted to Frontiers in Biosciences, 2008.
6. Kong M, Lee JJ. Applying Emax model and Bivariate Thin Plate Splines to Assess Drug Interactions. Submitted to Frontiers in Biosciences, 2008.
7. Behrens C, Lin H, Lee JJ, Hong WK, Wistuba, II, Lotan R. Immunohistochemical Expression of Basic Fibroblast Growth Factor and Fibroblast Growth Factor Receptors 1 and 2 in the Pathogenesis of Lung Cancer. Submitted, 2008.

Abstracts

1. Massarelli E, Maria L, Silva P, Ozburn N, Feng L, Yin G, O'Reilly M, Hong WK, Herbst RS, Wistuba II. Correlation between VEGF/VEGFR2 and EGFR immunohistochemical protein expression in early stage non-small cell lung carcinoma. The 98th AACR Annual Meeting, abstract#: 5029, 2007.
2. Sun M, Massarelli E, Feng L, Ozburn N, Yin G, Komaki R, Hong WK, Aldape KD, Wistuba II. Differential immunohistochemical expression pattern of HER family receptors and ligands is detected in primary lung cancers and corresponding brain metastases. The 98th AACR Annual Meeting, abstract #: 468, 2007.
3. Behrens C, Lin H, Lee J, Hong WK, Wistuba II, Lotan R. Differential immunohistochemical expression patterns of fibroblast growth factor-2, receptors 1 and 2, and syndecan-1 in squamous cell carcinoma and adenocarcinoma of the lung. The 98th AACR Annual Meeting, 2007.

Conclusions

Core B continued to provide statistical and data management support for all research projects in the IMPACT study and develop new methods of analyses.

Core C: Pathology Core

(Director: Ignacio Wistuba, M.D.)

The IMPACT interdisciplinary research proposal for studying targeted therapy of lung cancers has required extensive histopathologic, IHC, and molecular studies of cell and tissues specimens, which have been assisted, coordinated or performed by the Pathology Core. One of the most important roles of the Pathology Core has been to provide professional technical services for proper procurement, storage and use of human and animal tissues, as well as technical assistance for IHC analysis. In addition, the Pathology Core has provided assistance for collection and evaluation of tissue specimens in IMPACT clinical trials in lung cancer patients.

Aim 1 Develop and maintain repository of tissue, cell and serum specimens from patients with lung neoplasia, as requested by the various component projects.

Update

During 2007, two groups of tissue and cytology samples were collected for the different projects: a) Prospectively, frozen and corresponding archival tumor and normal lung cancer patients undergoing surgery in our institution and patients enrolled in the clinical trials; and b) Retrospectively, frozen and corresponding archival tumors and normal lung cancer patients' cases already banked in our Thoracic Malignancy (former SPORE)Tissue Bank in which extensive clinical data, including smoking history and survival information is available.

Project 1. For the IMPACT clinical trial using Erlotonib, chemotherapy (Tarceva^{R}) and radiotherapy in advanced non-small cell lung cancer (NSCLC) patients (Protocol 2005-1023; Principal Investigator: Dr. R. Komaki), the Pathology Core has developed a flow chart (Appendix A) to evaluate the quality of tissue tumor specimens for biomarker analysis, including *EGFR* mutations. We have screened samples from 3 patients. Methodologies for *EGFR* mutation, *EGFR* copy number by fluorescent *in situ* hybridization (FISH) and EGFR protein analyses have been already established in our Pathology Core.

Project 2. The aim designed to analyze HER and VEGF/R markers in human NSCLC tissue specimens was completed in 2006. During the past year, our set of tumor tissue specimens in TMAs from 330 NSCLC patients has been utilized to study additional angiogenesis-related markers, including HIF-1α and carbonic anhydrase IX (CA IX).

For the IMPACT clinical trial using ZD6474 in NSCLC patients with malignant pleural effusion (Protocol 2005-9029); Principal Investigator: Dr. C. Jimenez), we have collected processed and banked specimens from 7 patients. The samples stored are: a) frozen cell pellet; b) formalin-fixed and paraffin-embedded (FFPE) cell pellets blocks and histology sections; c) alcohol-fixed and frozen cell smears; e) cell pellets frozen in RNA later; f) cell pellets frozen in DMSO 12%.

Project 3. The aim designed to analyze GRP78, IL-11R and Eph5A markers in human NSCLC tissue specimens was completed (see Project 3 and Annual Report 2006).

Project 4. The aim designed to analyze bFGF, receptors FGFR1 and 2 markers in human NSCLC tissue specimens was completed (see Project 4 and Annual Report 2006).

Project 6. We prospectively collected fresh NSCLC tumor tissue specimens which have been distributed to Dr. Mao's laboratory for developing mouse tumor heterotransplants. In all cases, FFPE human tissue specimens have been collected and banked.

We extracted DNA from 80 NSCLCs, including normal and tumor tissue, and provided these samples to Dr. Krahe's laboratory for methylation analysis of TCF21 gene. Our set of NSCLC tumor tissue TMAs has been also utilized for immunohistochemical analysis of TC2F1 protein.

Aim 2 Develop innovative tissue and cell reagents from lung cancer patients for the investigation and validation of the molecular endpoints relevant to each component project.

Update

The development of new tissue and cell reagents from lung cancer patients by this Core was focused in four different methodologies:

a. Tissue and cell pellets microarrays (TMA). The TMAs prepared last year (primary lung tumors, paired primary and brain metastasis NSCLC tumors, and cell lines pellets) have been utilized for IHC and FISH analyses (see below). The clinico-pathologic data, including recurrence free and overall survivals have been recently updated.

b. Establishment of short-term cultures and cancer cell lines of clinical lung tumor specimens. The Pathology Core tissue culture facility has been setup. A repository of 50 NSCLC cell lines obtained from our DoD grants collaborators, Drs. John Minna and Adi Gazdar, UT-Southwestern Medical Center, Dallas, TX, has been established. In addition, primary cell cultures from NSCLC pleural fluids have been established from 4 patients. Methodology to culture cells from pleural fluid and tumor tissue specimens from NSCLC after freezing of specimens using DMSO has been established.

c. Lung cancer heterotransplants using clinical lung tumor samples. In collaboration with Dr. Mao's lab (Project 6), we have collected fresh NSCLC (adenocarcinoma and squamous cell carcinoma histology) tumor specimens from surgically resected tumors from the pathology frozen room at MDACC to establish tumor heterotransplant in nude mice. Fourteen of such heterotransplants have been established and tissue histopathological and IHC characterization is in progress.

d. Pleural fluid specimens. In collaboration with Dr. C. Jimenez, Department of Pulmonary Medicine, who is the Co-principal investigator of the IMPACT ZD6474 in NSCLC patients with pleural fluid (protocol), we have established a repository of 250 specimens of malignant pleural fluids, including 80 NSCLC cases (Table 2). Of these, 9 specimens correspond to patients enrolled in the IMPACT clinical trial. For each pleural fluid sample, we have obtained and stored the following (Table 2): a) frozen cell pellet; b) formalin-fixed paraffin-embedded (FFPE) cell pellets blocks and histology sections; c) alcohol-fixed and frozen cell smears; d) cell pellets frozen in RNA later; e) cell pellets frozen in DMSO 12%; f) frozen supernatant. We have optimized methodologies for IHC and FISH using the FFPE cell pellets obtained from malignant pleural effusions, so we will be able to analyze the samples obtained from IMPACT ZD6474 clinical trial.

Table 2. Summary of malignant pleural effusion samples collected and banked by the Pathology Core.

Sample Type	Lung Cancer			Other Tumors
	Adenocarcinoma (30 Cases)	Squamous Cell Carcinoma (11 Cases)	Small Cell Carcinoma (4 Cases)	(N=75)
Cryopreserved Cell Pellets	130	30	16	109
FFPE Block	49	11	4	59
Smears	88	22	5	95
Cell Pellet in RNA later	41	11	4	57
Supernatant	80	22	8	116

Aim 3 Process human and animal cell and tissues for histopathological, immunohistochemical (IHC) and molecular analyses, including tissue microdissection, as required by each component project.

Update

Tissue Processing. Archival and prospectively collected frozen and FFPE cell and tissue specimens from NSCLC patients have been processed and distributed for Projects 1, 2, 3, 4 and 6. Processing (cutting and H&E staining) for histology evaluation and sectioning for IHC have been performed in about 400 paraffin-embedded blocks from lung cancer, including TMA and whole tissue block specimens. Frozen lung cancer tissues and corresponding archival FFPE specimens, as well as tDNA extracted from tumor tissues, have been processed and provided for Project 6.

Aim 4 Perform and evaluate IHC analysis in human and animal cell and tissue specimens, as required by the various component projects.

Update

Collaboration with research projects during 2007. Examination of IHC markers in collaboration with IMPACT research projects has been the most important activity of the Pathology Core during 2007.

Project 2. Four main projects have been performed in the Pathology Core in collaboration with Project 2.

Completed Projects:

a) Correlation between VEGF/VEGFR2 and EGFR IHC protein expression in early stage NSCLC. In collaboration with Dr. R. Herbst (Co-PI Project 1), we showed that VEGFR and EGFR pathways are positively correlated in early stage NSCLC and IHC expression of p-VEGF-R2 is an indicator of worse overall survival in stage I-IIIA NSCLC. These results were presented in the 98th AACR Annual Meeting (Los Angeles, CA, April 2007) and a manuscript is in preparation by Ermina Massarelli et al.

b) *KRAS* Mutation is an important predictor of resistance to therapy with EGFR tyrosine kinase inhibitors (TKI) in NSCLC. In collaboration with Dr. R. Herbst (Co-PI Project 1), we showed that *KRAS* mutation represents a marker of resistance of NSCLC to EGFR-TKIs and should be included in the panel of markers used to predict response to such therapy. A paper has been published in Clinical Cancer Res by Massarelli et al., 2007.

c) Histopathologic and IHC analysis of NSCLC xenograft mice models to investigate correlation between activated EGFR (pEGFR) expression and molecular imaging using NSCLC cell lines. In collaboration with Dr. J. Gelovani (Co-PI Project 1), we showed that there is a reduction in the pEGFR membrane IHC expression in NSCLC cell lines xenograft tumor specimens in mice treated with EGFR tyrosine-kinase inhibitor, and this reduction of expression in tissues correlated with pEGFR-based *in vivo* imaging.

Ongoing Projects:

a) Analysis of the IHC expression of angiogenesis-related markers HIF-1α and carbonic anhydrase IX (CA IX) in tumor specimens from 330 NSCLC patients using TMAs. Both IHC methodologies have been optimized, the TMAs immunostained and the analysis performed

using image analysis. Statistical analysis to determine frequency of expression in tumors and correlation with patients' clinico-pathologic characteristics is in progress.

Project 3. Two main projects have performed and completed in the Pathology Core in collaboration with Project 2.

Completed Projects:

a) Analysis of IHC expression of GRP78 and IL-11R in NSCLC tissues obtained from surgically resected lung cancer specimens. In collaboration with Dr.R. Pasqualini (PI, Project 3), we have showed that both markers frequently overexpress in cytoplasm (GRP78 mean score 200 and IL-11R 125, range 0-300) and membrane (GRP78 80% and IL-11R 25%) of NSCLC tumor cells, so they represent potential targets for therapy in lung cancer.

b) Study of IHC expression of EphA5 in NSCLC tissues obtained from surgically resected lung cancer specimens. In collaboration with Dr. R. Pasqualini (PI, Project 3), we have showed that EphA5 frequently expresses in cytoplasm (mean score 130, range 0-300), membrane (40%) and nucleus (70%) of NSCLC tumor cells, so also represents a potential targets for therapy in lung cancer.

Project 4. Differential IHC expression patterns of bFGF, receptors 1 and 2, and syndecan-1 in NSCLC. This study corresponds to Aim 1 of Project 4 and it was completed in 2006. An abstract was presented in the 98th AACR Annual Meeting (Los Angeles, CA, April 2007) and a paper has been submitted to Clin Cancer Res by Carmen Behrens et al.

Project 6. Two projects have performed in the Pathology Core in collaboration with Project 6.

Completed Project

a) Analysis of IHC expression of TCF21 in NSCLC tissues obtained from surgically resected lung cancer specimens using TMAs. In collaboration with Dr. R. Krake (Co PI Project 6), we studied 300 NSCLC in TMAs for the expression of the protein TCF21, a putative tumor suppressor gene located in chromosome region 6q23-24. We showed that TCF21 protein is significantly reduced or lost in 166/191 (87%) of adenocarcinomas and 87/109 (80%) of squamous cell carcinomas (Table 3 and Figure 18). Figure 24 is representative of expression detected in NSCLC cells by TMA for TC21.

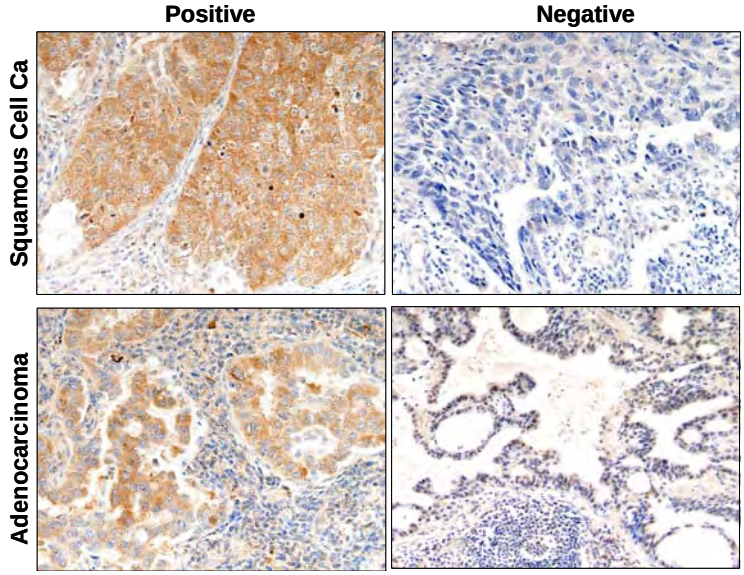
Table 3. Frequency of TCF21 protein reduction or loss in NSCLC tumor tissue specimens.

Characteristics	Negative (N=98) Score 0-9		Low (N=155) Score 10-99		Intermediate (N=39) Score 100-199		High (N=8) Score 200-300	
	n	%	n	%	n	%	n	%
Tumor Histology								
Adenocarcinoma	70	71.4%	96	61.9%	24	61.5%	1	12.5%
Squamous Cell Ca	28	28.6%	59	38.1%	15	38.5%	7	87.5%
Tobacco History								
Never	20	20.4%	25	16.1%	6	15.4%	2	25.0%
Ever	78	79.6%	130	83.9%	33	84.6%	6	75.0%
Pathological Stage								
I	59	60.2%	101	65.2%	24	61.5%	5	62.5%
II	20	20.4%	28	18.1%	7	17.9%	3	37.5%
III	14	14.3%	23	14.8%	7	17.9%	0	0.0%
IV	5	5.1%	3	1.9%	1	2.6%	0	0.0%

Ongoing Project:

a) Study of IHC expression of protein Twist in NSCLC tissue specimens, using TMAs. In collaboration with Dr. R. Krake (Co-PI, Project 6), we have optimized the IHC conditions of Twist antibody, a protein associated to epithelial mesenchymal transition (EMT) phenomenon, using FFPE tissues. The staining of the TMA set containing 330 NSCLC tumor tissues with annotated clinico-pathologic data is in progress.

Figure 24. Representative examples of TCF21 immunohistochemical expression in lung tumors. In left panel, two tumors showing strong cytoplasmic expression. In right panel, two tumors showing lost of expression.



<u>Additional IMPACT research activities of the Pathology Core</u>. The Pathology Core has performed independent research activities to better characterize the *EGFR* molecular abnormalities involved in: **a) the progression of lung cancer**, **b) its correlation with other lung cancer molecular abnormalities**, and **c) to establish their role in the development of lung cancer brain metastasis**. These studies correlate with ongoing research activities in the corresponding IMPACT research projects (especially Projects 1 and 2). As a result of this additional work performed by the Pathology Core, two papers have been published and four manuscripts are in preparation. In addition, two abstracts were presented in the 2007 AACR Annual Meeting (Los Angeles, CA, April 2007) and four abstracts have been accepted for presentation in the 2008 AACR Annual Meeting (San Diego, CA, April 2008).

Completed Projects:

a) EGFR abnormalities in the progression of lung adenocarcinomas. Using a detailed molecular pathology mapping strategy, we investigated the frequency and characteristics of EGFR abnormalities (mutation, copy number and protein expression) in primary tumors and corresponding lymph node metastases, obtained from 24 lung adenocarcinoma specimens with *EGFR* mutation. We concluded that higher levels of EGFR overexpression and more homogeneously distributed high gene copy numbers are present in metastasis than corresponding primary tumors. Our findings have important implications for the development of new strategies for targeted therapy in lung adenocarcinoma using EGFR inhibitors. This data are part of a recently submitted paper to Cancer Prevention Research (CPR) by Ximing Tang et al.

b) Association of EGFR gene abnormalities with other molecular abnormalities in NSCLC. We have examined the association of three different pathways with EGFR abnormalities in lung cancer:

b.1. Estrogen and progesterone receptors expression. Estrogen (ER) α and β and progesterone (PR) receptors are transcription factors that regulate the expression of multiple

genes and have been involved in the pathogenesis of non-small cell lung cancer (NSCLC). We have demonstrated that both ERs (α and β) and PR frequently express in NSCLC. Importantly, we identified antibodies against ERs that distinguish a subset on NSCLC having defined clinico-pathologic features, including *EGFR* mutation in lung adenocarcinoma histology. Higher nuclear and cytoplasmic expressions of ER α antibodies clones HC-20 and 1D5N, and higher nuclear expression an ERβ antibody clone H150 correlated with the presence of *EGFR* mutation in adenocarcinomas (p = 0.0029 to <0.00001). These data will be presented in the 2008 AACR Annual Meeting (San Diego, CA, April 2008) and a manuscript is in preparation by Gabriela Raso et al.

b.2. NKX2-1 (TITF-1) gene amplification and protein overexpression. Using support of our Department of Defense grants VITAL and IMPACT in 2006, we participated in a Lung Adenocarcinoma Consortium to identify novel genes amplified or deleted in lung adenocarcinomas by analyzing chromosomal loci copy numbers using single nucleotide polymorphism (SNP)-array analysis. We showed that *NKX2-1* gene (also known as thyroid transcription factor-1, TITF-1), a lineage-specific transcription factor frequently overexpressed in lung adenocarcinoma, is amplified in a subset (12%) of these tumors. Then, we have demonstrated that *NKX2-1* (TITF-1) amplification occurs in a subset of both major types of NSCLCs histologies: adenocarcinoma (19%) and squamous cell carcinoma (18%). We showed that gene amplification correlated with protein overexpression in adenocarcinomas. NKX2-1 protein expression correlated with *EGFR* mutation (P<0.001). Survival analysis showed that for adenocarcinoma *NKX2-1* amplification by FISH correlated with worse recurrence-free survival (p = 0.001), while IHC overexpression correlated with better recurrence-free survival (p = 0.036). These data will be presented in the 2008 AACR Annual Meeting (San Diego, CA, April 2008) and a manuscript is in preparation by Ximing Tang et al.

b.3. NOTCH3/JAGGED1 pathway. Notch3 (N3) is a member of the family of Notch transmembrane receptors, which are activated by ligands such as Jagged1 (JAG1). In addition to roles in normal cellular differentiation and cell-fate determination, the Notch pathway is involved in tumorigenesis. N3 is differentially expressed in lung cancer and sensitizes tumors to EGFR inhibition. However, the relationship of N3 and JAG1 protein expression with EGFR has not been studied in lung cancer. Our findings indicate that N3-JAG1 proteins are frequently expressed NSCLC. N3 amplification is present in 8% of tumors and is associated with smoking history and squamous cell differentiation. N3 protein levels in ADCs correlated with the presence of EGFR mutations. These findings suggest that NSCLC is commonly associated with aberrations in the N3-JAG1 pathway. These data will be presented in the 2008 AACR Annual Meeting (San Diego, CA, April 2008) and a manuscript is in preparation by Ludmila Prudkin et al.

c) Characterization of HER family receptors markers and EGFR gene abnormalities in NSCLC primary tumors and brain metastasis. Using 60 paired NSCLC primary and corresponding brain metastasis tumor tissues, we demonstrated that there is differential expression pattern of HER family receptors and ligands expression in both tumors' sites. We showed that Amphiregulin, pHer3 and pEGFR are significantly overexpressed in brain metastasis sites. These data were presented in the 2007 AACR Annual Meeting (Los Angeles, CA, April 2007). Recently, we have shown that NSCLC brain metastasis sites (68%) and their corresponding primary tumors (59%) demonstrate a very high frequency of *EGFR* increased copy number by FISH, especially gene amplification (brain metastasis 29%; primary tumors 17%), suggesting that this molecular abnormality may be involved in tumor progression and metastasis phenomenon. These data will be presented in the 2008 AACR Annual Meeting (San

Diego, CA, April 2008). A manuscript having both set of data is in preparation by Menghong Sun et al.

Key research accomplishments

- Developed a repository of lung cancer tissue, cytology and cell lines specimens with annotated clinical data, to be utilized for research projects and helped to develop a series of lung cancer heterotransplants in mice in collaboration with Project 6.
- Characterized the expression of angiogenic markers (VEGF/VEGFR and bFGF/Receptors), HER family receptors, and tumor cell membrane/cytoplasmic proteins (GRP78, IL-11R and EphA5) in NSCLC, and established their correlation with clinicopathologic features
- Identified the role of EGFR abnormalities in the progression and metastasis of lung adenocarcinomas, and established the correlation with other important cancer pathways, such as ER/PR, NOTCH and NKX2-1.

Reportable outcomes

Two abstracts presented in the 2007 AACR Annual Meeting (Los Angeles, CA, April 2007) and reported last year, four abstracts accepted for presentation in the 2008 AACR Annual Meeting (San Diego, CA, April 2008) in which the Pathology Core PI (I. Wistuba, M.D.) is the senior author. One paper has been published, two submitted and four are in preparation.

Manuscripts published in peer-reviewed

1. Weir BA, Woo MS, Getz G, Perner S, Ding L, Beroukhim R, Lin WM, Province MA, Kraja A, Johnson L, Shah K, Thomas RK, Barletta JA, Borecki IB, Broderick S, Chang A, Chiang DY, Chirieac LR, Cho J, Fujii Y, Gazdar A Giordano T, Greulich H, Johnson BE, Kris MG, Lash A, Lin L, Lindeman N, Mardis ER, McPherson JD, Minna J, Morgan MB, Nadel M, Orringer MB, Osborne JR, Ozenberger B, Ramos AH, Robinson J, Roth JA, Rusch V, Sasaki H, Sato M, Shepard F, Spitz MR, Tsao M-S, Twomey S, Verhaak R, Weinstock GM, Wheeler DA, Winckler W, Yoshizawa A, Yu S, Zakowski MF, Zhang Q, Beer DG, **Wistuba I**, Watson MA, Garraway LA, Ladanyi M, Travis W, Pao W, Rubin MA, Gabriel S, Gibbs R, Varmus H, Wilson RK, Lander E, Meyerson M. Characterizing the cancer genome in lung cancer. Nature, 450:893-898, 2007.

Manuscripts in press, review, revision, or submission

1. Behrens C, Lin H, Lee JJ, Hong WK, Wistuba, II, Lotan R. Immunohistochemical Expression of Basic Fibroblast Growth Factor and Fibroblast Growth Factor Receptors 1 and 2 in the Pathogenesis of Lung Cancer. Submitted, 2008.
2. Tang X, Varella-Garcia M, Xavier AC, Massarelli E, Ozburn N, Moran C, Wistuba II. EGFR Abnormalities in the Pathogenesis and Progression of Lung Adenocarcinomas. Submitted to Cancer Research Prevention.

Abstracts

1. Massarelli E, Prudkin Silva ML, Ozburn N, Feng L, Yin G, O'Reilly M, Hong WK, Herbst RS, Wistuba II. Correlation between VEGF/VEGFR2 and EGFR immunohistochemical protein expression in early stage non-small cell lung carcinoma. The 98th AACR Annual Meeting, abstract#: 5029, 2007.
2. Sun M, Massarelli E, Feng L, Ozburn N, Yin G, Komaki R, Hong WK, Aldape KD, Wistuba II. Differential immunohistochemical expression pattern of HER family receptors and ligands is

detected in primary lung cancers and corresponding brain metastases. The 98th AACR Annual Meeting, abstract #: 468, 2007.

2008 (Submitted and Accepted)

1. Raso MG, Behrens C, Liu S, Prudkin L, Denise M. Woods, Natalie Ozburn, Cesar Moran, J. Jack Lee and Ignacio Wistuba. Immunohistochemical expression of estrogen and progesterone receptors identifies a subset of non-small cell lung cancers and correlates with EGFR mutations. Accepted for poster presentation in the 2008 AACR Annual Meeting (San Diego, CA, April 2008).
2. Tang X, Sun M, Behrens C, Prudkin L, Ozburn N, Gazdar AF, Moran C, Varella-Garcia M, Wistuba II. TTF-1 gene amplification and protein expression pattern identify adenocarcinoma of lung with worse prognosis. Accepted for platform presentation in the 2008 AACR Annual Meeting (San Diego, CA, April 2008).
3. Prudkin L, Liu D, Tchinda J, Woods D, Behrens C, Bekele BN, Moran C, Lee C, Aster JC, Zhou B-B, Wistuba II. NOTCH3/JAGGED1 pathway is involved in non-small cell lung cancer pathogenesis and interacts with EGFR pathway. Accepted for poster presentation in the 2008 AACR Annual Meeting (San Diego, CA, April 2008).
4. Sun M, Massarelli E, Ozburn N, Tang X, Prudkin L, Komaki R, Hong WK, Aldape KD, Moran C, Varella-Garcia M, Wistuba II. *EGFR* increased copy number is frequent in non-small lung cancer with brain metastasis. Accepted for poster presentation in the 2008 AACR Annual Meeting (San Diego, CA, April 2008).

Conclusions

The Pathology Core has assisted and collaborated actively with several research projects performing multiple histopathological, immunohistochemical and molecular studies in a large series of lung cancer tissues specimens. In addition, the Pathology Core has managed to conduct specific research activities, which fully integrate with some of the IMPACT research projects. Because direct contribution of the Pathology Core, two abstracts have been presented in international meetings and 4 accepted for presentation, a paper has been published in *Nature*, two submitted and four are in preparation for submission. The Pathology Core has successfully fulfilled the goals proposed for the third year of IMPACT project.

Core D: Imaging Core: Provide Imaging Support for IMPACT Projects

(PI and co-PI: Juri Gelovani, M.D., Ph.D.; Chun Li, Ph.D.)

Project 2. During the third year of funding of Project II, the work in the Imaging Core was focused at the Aim 3 – synthesis and 18F-radiolabeling of the IV-generation of active-mutant EGFR agent for PET imaging. The Core has synthesized a series of IV-generation compounds with oligo-PEG side chain of different length (2, 4, and 6 ethylene glycols in chain) in the position 7 of quinazoline moiety. The Core developed and optimized the radiolabeling procedures for the routine synthesis of ^{18}F-PEG6-IPQA, which was selected by the leaders of Project II, based on its selectivity to L858R active mutant EGFR and good water solubility (Figure 25).

Imaging Core has conducted all microPET imaging studies reported in Project 2.

Towards the end of this reporting period, Imaging Core initiated the development and evaluation of standard operating procedures and chemistry, manufacturing, and control section of an IND for Phase I clinical study on PET imaging with ^{18}F-PEG6-IPQA to assess radiation dosimetry and radiolabeled metabolites in patients. Also, the Imaging Core has developed a scale-up procedures for synthesis of precursor for radiolabeling of ^{18}F-PEG6-IPQA, to decrease the cost associated with setting up and scaling up procedures at the CGMP manufacturing site (Macrocyclics, Inc., Dallas, TX).

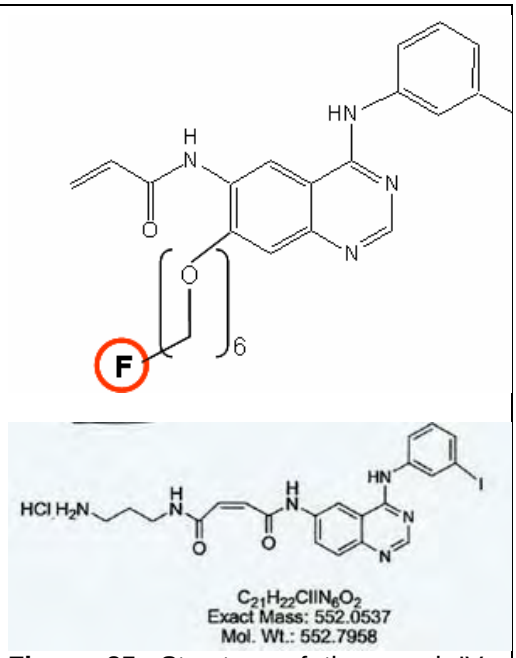


Figure 25. Structure of the novel IV-generation agent ^{18}F-PEG6-IPQA with selectivity for active mutant EGFR and improved water solubility (top) in comparison to the III-generation compound JGAP5 (bottom).

Project 4. Imaging Core has conducted bioluminescence imaging (BLI) studies in mice bearing s.c. xenografts of A549 carcinomas expressing GFP-Luc (GL) dual-modality reporter gene (previously transfected by the Imaging core). The expression of GL reporter gene in A549GL tumors growing in mice was assessed by BLI after i.p. injection of D-luciferin (Biosynth, Int., Naperville, IL) substrate at 150 mg/kg per mouse 10 minutes prior to imaging with IVIS200 system (Xenogen, Almeda, CA).

Key Research Accomplishments

- Maintained SOPs and performed QC/QA procedures for routine synthesis of ^{111}In-DTPA-PEG-AnnexinV for SPECT/CT imaging in Project I (however, no imaging studies have yet been requested from the Imaging Core by investigators of this project).
- Produced novel ^{18}F-PEG6-IPQA derivative for PET and in vitro uptake studies for Project II;
- Performed all multi-modality imaging (and autoradiography) studies in mice with orthotopic models of different NSCLC using ^{18}F-PEG6-IPQA (EGFR expression/activity), ^{18}F-FMAU (proliferative activity), and ^{18}F-FDG (glucose metabolism) in Project II.
- Performed routine synthesis of [^{18}F]FEAU for Project III and conducted microPET imaging studies for this project.
- Performed synthesis and ^{111}In / ^{64}Cu radiolabeling of GRP78-specific cyclic nanopeptide CGRRAGGSC for imaging studies in Project III for imaging studies with ^{64}Cu-DOTA-CGRRAGGSC PET/CT in animals for Project III.
- CGMP production of precursor for 18F-radiolabeling of [^{18}F]-PEG6-IPQA has been subcontracted to CGMP qualified company (Macrocyclics, Inc., Dallas, TX) for the future Phase I clinical study.

Reportable Outcomes

Resources

Radiolabeled compounds available for use by projects: ^{18}F-PEG6-IPQA (EGFR expression/activity), ^{18}F-FMAU (proliferative activity), and ^{18}F-FDG (glucose metabolism), and ^{111}In / ^{64}Cu radiolabeling of GRP78-specific cyclic nanopeptide CGRRAGGSC.

Conclusions

The Imaging Core continues to provide imaging support requested by the PIs of IMPACT research projects, in addition to synthesizing more optimal IPQA derivatives.

DRP-1: Treatment of Malignant Pleural Effusion with ZD6474, a Novel VEGFR and EGFR TK Inhibitor

(PI and co-PI: Roy Herbst, M.D., Ph.D., Carlos Jimenez, M.D.)

Aim 1 To determine clinical effect of ZD6474.

Aim 2 To investigate biological correlates.

Aim 3 To investigate radiographic correlates.

Aim 4 To assess quality of life.

Update

The amended single arm, open-label study to evaluate the efficacy of ZD6474 on the management of pleural effusion in NSCLC patients was activated in June of 2007.

To date, 7 patients have been enrolled and treated. Three additional patients were enrolled but did not received medication (one non-compliance, one with a benign pleural effusion and one with a loculated effusion not amenable for intrapleural catheter placement).

Days with intrapleural catheter in place. Five patients have had their intrapleural catheters removed at days 13, 14, 15, 22 and 28. One patient remained with the intrapleural catheter after 10 weeks of treatment and it was removed 142 days after insertion. The last patient was enrolled recently and data is pending.

Weeks on study and related adverse events. Three patients stopped medication at week 6 (two) and 7 due to grade 3 related adverse events (two QTc prolongation and one fatigue). Two patients completed ten weeks of treatment and one took the medication for 18 weeks. The last patient was recently enrolled and data is pending.

Best response: Three patients had stable disease, one patient had a partial response and two patients had disease progression. The last patient was recently enrolled and data is pending.

Correlative studies. In collaboration with Dr. Wistuba, Core C Director, we have been collecting pleural effusion specimens including cryopreserved cell pellets, supernatants, FFPE blocks, smears, and RNA for correlative studies (see Table 2, Core C).

Key Research Accomplishments

- Trial was approved and activated.
- Almost one third of the total sample size (25) has been enrolled.
- Collected 116 specimens for correlative analyses.

Reportable Outcomes

None at this time.

Conclusions

The trial is activated and well underway; all analyses will be performed after the trial is completed.

DRP-2: TALK - Teens and Young Adults Acquiring Lung Cancer Knowledge

(PI: Alexander V. Prokhorov, M. D., Ph.D.)

Ninety percent of lung cancer cases in adults are direct results of smoking. In children and young adults tobacco use remains a major public health problem in spite of the recent declines in smoking prevalence among children and adolescents. Over the past 2-3 decades, numerous factors of smoking initiation among adolescents have been thoroughly investigated. A considerable volume of literature is currently available providing important clues with respect to designing tobacco prevention and cessation among youth.

Focusing on this major public health problem – tobacco use among young individuals and lack of in-depth knowledge of lung cancer issues – Project TALK (Teens and Young Adults Acquiring Lung Cancer Knowledge) was conceived and funded as a smoking cessation/prevention pilot project for culturally diverse high-risk young populations that include school drop-outs, economically disadvantaged, and underserved. Using modern technologies, the Departments of Behavioral Science and Thoracic/Head & Neck Medical Oncology have joined their efforts to conduct this developmental project under the leadership of Dr. Alexander V. Prokhorov. The project will assist in making major advances in lung cancer education and prevention among youth. Project TALK will produce a CD-ROM-based education/behavior change for teenagers and young adults (15-24 years of age).

We have thus been devoting our effort in 4 tasks as described in the Statement of Work based on the project timeline:

Task 1. **Develop intervention program.** Focus groups will be held with adolescents and young adults to ensure we are capturing the essence of the program, using the right messages, and employing the appealing video and animated characters. (Years 1-2)

Task 2. **Develop and beta-test CD-ROM.** This includes the design of the animation, illustrations, scripts and accompanying videos. (Years 1-2)

Task 3. **Implement program in agreed upon locations and recruit young adults to participate in the study.** (Years 3-4)

Task 4. **Collect and analyze data.** (Year 3-4)

Update

Years 01-02 were devoted to intervention program conceptualization and development. Beginning in 2007 (Year 03), the program began implementation of the testing phase among an ethnically diverse sample of youth to evaluate its feasibility and impact.

TALK Deployment Location: To ensure that the target population would be accessed for project TALK, Dr. Alexander V. Prokhorov met with Dr. Lovell Jones from the Department of Health Disparities Research at The University of Texas M. D. Anderson Cancer Center to discuss possible game operating venues. Dr. Jones suggested several potential locations. We contacted some and decided to work with Sharpstown Mall, billed as Houston's Premiere Urban Mall. A general agreement has been signed and submitted for recruitment to begin the third week of March 2007.

Project members Julie John and Chris Strachan went out to Sharpstown Mall multiple times in April and May 2007 to finalize recruitment but due to the hazardous environment at Sharpstown Mall the staff decided there were no safety measures to ensure the safety and well being of project members.

Recruitment of three new locations took place in May 2007 at Grace Community Center, 5th Ward Enrichment Center and M.R. Wood, an alternative education program. Recruitment began in October 2007 at 5th Ward Enrichment Center, followed by recruitment at M.R. Wood beginning in November 2007. The Fort Bend County Juvenile Justice Program is another alternative education program within M.R. Wood. Recruitment from the Fort Bend County Juvenile Justice Program took place from January 2, 2008 to January 16, 2008.

Contact: Each target site has a contact person that has assisted in the implementation of Project TALK. At the 5th Ward Enrichment Center Mr. Charles Savage, Project Director is the contact and person assisting in recruitment. Mrs. Irma Longoria, school counselor specializing in gang relations and tobacco education is the primary contact who has also been instrumental in the project's recruitment and implementation.

Participant Recruitment: A total of 153 participants have been recruited from two locations, 5th Ward Enrichment Center and M. R. Wood. From 5th Ward Enrichment Center 3 African-American male participants were recruited.

From M.R. Wood 150 participants were recruited. Out of the150, 126 are male and 24 female; 75 are African-American, 60 are White, 9 are Asian, 3 are American Indian/Alaskan Native, 2 are Native Hawaiian or Other Pacific Islander and 1 is Other.

The recruitment process at 5th Ward Enrichment Center was assisted by the program Executive Director and at M.R. Wood a school counselor assisted in recruiting the participants. At M.R. Wood the students were notified of the project by the school counselor and incoming students were given orientation packages that included the informed consent form that was then turned in to the teaching staff or to the counselor. Other students were recruited by Project TALK staff through informal interactions with students who had heard about the game and were interested in participating.

A total of 136 participants have completed 7 day follow ups. The remaining participants which are due to complete 7 day follow ups are currently in progress. Six month follow ups will be collected in April 2008. Because data collection is still underway, no extensive feasibility or efficacy results are presented in this program report. Please see Implentation Schedules in Appendix B.

Testing: Testing of Project TALK at the 5th Ward Enrichment Center was performed in a large conference room at the center. The participants were given a short introduction to the study after turning in their signed informed consent forms to staff members. The participants were then asked to complete a contact information sheet and a baseline survey. Afterwards, the participants played the kiosk version of the educational videogame for 20 minutes, at the end of 20 minutes they were asked if they wanted to play for another 10 minutes. At the end of this session, the participants were given a CD-ROM version of the game to take home and use it at their leisure.

Mr. Charles Savage arranged for the participants of the program to be transported back to the target site to complete the 7-day follow-up survey. The survey was completed in the same large conference room. After the completion of the survey the participants receive their incentive gift cards and are reminded of their 6-month follow-up survey which will be conducted over the phone.

At M.R. Wood, student's schedules were coordinated such that no more than two participants at a time played the kiosk version of the videogame. In the conference room, the students were given a brief introduction to the study and completed a contract information sheet as well a baseline survey. After completing the survey the participants used the kiosk version of the videogame for 20 minutes. At the end of 20 minutes they were given the option of playing an additional 10 minutes.

During the collection of the 7-day follow-up survey, the same routine is followed from getting the participants' schedules, getting them from class and taking them to the small conference in order to complete their survey. No more than four participants complete their 7-day follow-up surveys at a given time in the small conference room. After the completion of the survey the participants receive their incentive gift cards and are reminded of their 6-month follow-up survey which will be conducted over the phone.

Testing of this highly innovative educational tool is currently in progress. The testing is anticipated to be completed by May 2008.

Survey: The evaluation instruments of the program have been designed and consist of a baseline survey and 7-day follow-up survey, both of which have been used in target sites. The 6-month follow-up survey has also been designed and will be reviewed before its use out in the target testing areas in April 2008.

Baseline Survey:

The baseline survey consists of sociodemographic questions such as:

- Date of birth
- Gender
- Ethnic/racial background

Other questions involve educational items:

- Are you in school?
- What is the highest grade/year you completed in school?
- How often do you skip school?
- What kinds of grades do you get in school?
- During this school year, how many times have you had detention?
- During this school year, how many times have you had suspension?

The remaining baseline survey questions involve cigarette use:

- Does anyone who lives in your home smoke cigarettes?
- How many of your closest friends smoke cigarettes?

The next set of questions asks the participant to indicate their current smoking status and status of change by asking:

- Are you a smoker right now?

Current smokers are asked the following:

- On average, how many cigarettes do you smoke in one day?
- How long have you been smoking regularly?
- In the last year, how many times have you quit smoking for at least one day?
- Do you really want to quit smoking?

If on the other hand, if the participant indicates s/he is a nonsmoker the following questions are asked:

- If one of your best friends gave you a cigarette, would you smoke it?
- Do you want to try cigarette smoking in the next year?
- Do you want to try cigarette smoking in the next 6 months?
- Do you want to try cigarette smoking in the next 30 days?

The next set of questions involves knowledge, beliefs and attitudes about smoking. The participant is asked to rate how important each of the following statements are in their decision to smoke using a three point scale. The three point scale is 1= not important, 2= somewhat important and 3= very important. The statements include:

- Smoking makes kids get more respect.
- Smoking helps people deal with problems.
- Smoking cigarettes is bad for your health/makes you sick.
- Cigarette smoking can make people around you sick.
- Smoking cigarettes makes you chill/relax and cigarette smoking gets you hooked.

Following is a section of questions concerning self-efficacy and temptation to smoke. Again a three point scale is used for the participants to indicate how tempted they are to smoke in each situation. The three point scale is 1= not at all tempted, 2= somewhat tempted, and 3= very tempted. The situational statements include:

- With friends at a party
- When I first get up in the morning
- When I am very anxious and stressed
- Over coffee while talking and relaxing
- When I feel I need a lift
- When I am angry about something or someone
- With my spouse of close friend who is smoking
- When I realize I haven't smoked for a while
- When things are not going my way and I am frustrated

The last sets of questions are about health perception:

- How healthy are you?
- Is smoking making you sick?
- Do you think that a smoker can improve his health by quitting?

7-Day Follow-Up Survey:

The 7-day follow-up survey first asks:

- Within the last 7 days, how many times did you play the game?

The next question asks the participant to indicate how easy it was to play the game, specifically:

- The whole game
- Directions in the game
- Moving from one room to another
- To build the main character of the game

Next the participants are asked how much they liked the following:

- The colors and pictures used in this game
- The cartoons in this program
- The videos showing interviews with people
- The videos showing surgery
- The background music
- The hospital
- The people who helped you play the game.

The next question is:

- Which room in the game did you like the most?

The following questions ask about the involvement – motivational appeal/ educational relevance/ behavior change. A four point scale of strongly agree, agree, disagree and strongly disagree is used. The questions are:

- The game made we want to learn about smoking and why it is bad for health
- The educational materials made me know more about smoking and how it is bad for me.
- Playing this game has made me want to never start smoking/ stop smoking.
- This game helped me deal with my friend trying to get me to smoke.
- This game has made me worry about my family and friends who smoke.
- I have shared/plan to share this game with my family/friends.

Lastly, the following questions find out what the participant learned while playing the game; they indicate if the statements are true, false or if they don't know. The statements are as follows:

- Tobacco kills more people than alcohol, murder, cocaine, heroin and AIDS combined.
- Smoking cigarettes can't get you hooked like other drugs.
- Smoking makes you creative and smart.
- Tobacco smoke has 4000 chemicals and 43 can cause cancer in people.
- If you smoke for many years it can give you lung cancer and heart disease.
- It is OK to smoke because many movie stars smoke and still stay healthy.
- Smoking costs a lot of money, more than just the money you spend to buy them, it makes your health insurance more expensive, and can make you lose your job.
- Big tobacco companies try to get young smokers, because older people who smoke are dying.
- Smoking when you're pregnant does not hurt the baby.
- Smoking can cause damage to your health and your looks after a few months, like gum disease, wrinkles and even sex problems.
- Kids whose parents smoke around them can have a lot of health problems due to secondhand smoke.

Participants are then given the option to leave comments or suggestions with regard to their perception of the program and how it can be improved.

The intervention tool is completed and is currently being used for testing according to the study timeline. All educational modules are in place. The program underwent the first round of beta testing and all the glitches and problems were removed.

Key Research Accomplishments
- Protocol for testing videogame was approved and activated.
- Performed testing at two sites with a total of 303 participants.

Reportable Outcomes

Resources
The intervention tool is completed (kiosk and CD) and is currently being used for testing according to the study timeline. All educational modules are in place. The program underwent the first round of beta testing and all the glitches and problems were removed.

Conclusions
Project TALK is successfully developing according to the timeline. It produced an innovative, highly informative, and easy-to-navigate videogame which was enthusiastically accepted by young individuals involved in the pre-testing activities. The investigative team has begun testing and has moved to the evaluation phase of the study for which community venues have been identified and their support and cooperation secured. A preliminary analysis is expected in March 2008.

Career Developmental Project (CDP1): Identification of Membrane Proteins in Bronchial Epithelia Cells as Biomarkers of Early Detection for Lung Cancer

(PI: Shin-Myung Kang, M.D.)

Lung cancer is the leading cause of cancer deaths. Early detection of the malignant lesion leads to an improved 5-year survival rate after surgical resection. Therefore, advanced screening tools are needed urgently to detect lung cancer at an early stage to improve control of such deadly lung cancer.

Aim 1 To isolate membrane proteins uniquely expressed on the surface of squamous metaplasia using organotypically cultured bronchial epithelial cells.

Membrane proteins will be isolated from squamous metaplastic bronchial epithelial cells and compared with that of normal mucociliary bronchial epithelial cells by 2-dimensional polyacrylamide gel electrophoresis.

Update

1. Isolation of membrane proteins.
First, we optimized isolation conditions of membrane proteins using lung cancer cell lines, A549 and H1975. We assessed the membrane proteins on 2-D PAGE and compared the spots (Figure 26). These cells were chosen as well-characterized samples to perform optimization of the method.

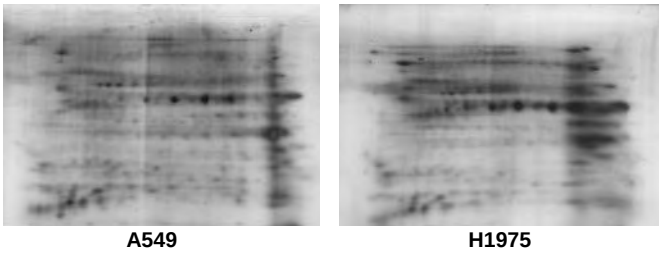


Figure 26. 2-D PAGE using membrane protein of A549 and H1975 cell lines. There is no significant difference between the cell lines on 2-D. (This was performed as an optimization process of the isolation of membrane protein and 2-D PAGE.)

2. Culture bronchial epithelial cells.
We have established organotypic culture system for primary bronchial epithelial cells and confirmed the development of squamous metaplasia cultured in media without retinoic acid. Now, we are culturing bronchial epithelial cells in air-liquid interface. Once a fully differentiated state showing normal mucocilliary or squamous metaplastic bronchial epithelial cells is reached, we will isolate membrane proteins from the cells for 2-D PAGE and for experiments in Aim 2.

Aim 2 To identify differentially represented proteins using proteomics.

Update

Using HPLC-tandem Mass Spectrometer in collaboration with the Proteomics Core facility in our institute, we will sequence and determine the specific identity of the proteins isolated in Aim 1. After isolation of membrane protein from normal mucocilliary and metaplastic bronchial epithelial cells, we will proceed with this analysis.

Aim 3 To verify the differentially represented proteins using PCR, Western blotting, and immunocytochemistry.

Update

Further verification will be done by real-time PCR, Western blotting analysis, and IHC analysis of the putative biomarkers on lung cancer specimens in TMAs in collaboration with the

Pathology Core. In addition to verification using clinical samples, we will perform histologic analysis and collect RNA and cell lysate from normal and metaplastic bronchial epithelial cells cultured in air-liquid interface.

Key Research Accomplishments

- Began organotypic cell culture for human bronchoepithelial cells and differentiation into squamous metaplasia.
- Optimized 2-D PAGE method.

Reportable Outcomes

None at this time.

Conclusions

This career development project has just begun. It has enormous potential for interesting proteomic results on tumor development which may be developed into early screening tools as well as for training and career development.

KEY RESEARCH ACCOMPLISHMENTS

Project 1: Targeting epidermal growth factor receptor signaling to enhance response of lung cancer to therapeutic radiation.

- Completed regulatory review of the clinical protocol for erlotinib plus chemoradiation which was approved by all agencies. This trial has been activated and patient accrual has begun.
- Demonstrated that, similar to the case of gefitinib, erlotinib also suppresses radiation-induced activation of pERK downstream of EGFR.
- Completed our investigation, using the neutral comet assay and pulsed field gel electrophoresis, showing that gefitinib suppresses the repair of radiation-induced DNA double strand breaks in NSCLC cells and submitted our findings for publication.
- Demonstrated that gefitinib may radiosensitize by inducing cells to express pNBS1 abnormally.
- Showed that the c-Met inhibitor, SU11274, radiosensitizes NSCLC cells presumably by suppressing the compensatory signaling that is responsible for resistance to radiosensitization by gefitinib.
- Initiated animal studies.

Project 2: Molecular imaging of EGFR expression and activity in targeted therapy of lung cancer

- It is noteworthy, that the hepato-billiary clearance of the ^{18}F-PEG6-IPQA radioactivity was further reduced as compared to the previous two generations of compounds - [^{124}I]-JGAP5 and [^{124}I]-mIPQA, which explains (at least in part) the reason for higher accumulation of water-soluble [^{18}F]-PEG6-IPQA in tumor tissue, which is due to increased clearance half-time and the AUC (input function) in blood.
- A significantly higher tumor-to-lung accumulation ratio (>10-fold contrast) was observed at 24 hours after i.v. administration of ^{18}F-PEG6-IPQA, which was higher than that observed with [^{124}I]-mIPQA (about 6-7 fold).
- Most importantly, we have developed a novel agent which is highly selective for L858R dominant active mutant EGFR and can provide highly specific PET imaging results.
- Pre-IND pharmacokinetics and radiation dosimetry studies for [^{18}F]-PEG6-IPQA are underway in non-human primates.
- Pre-IND acute toxicology studies are underway and are subcontracted to a company (Charles River Labs)
- CGMP production of precursor for 18F-radiolabeling of [^{18}F]-PEG6-IPQA has been subcontracted to CGMP qualified company (Macrocyclics, Inc., Dallas, TX).

Project 3: Targeted peptide-based systemic delivery of therapeutic and imaging agents to lung tumors

- Demonstrated specificity of phage for EphA5 receptor.
- Determined that phage expressing these peptides selectively localize to tumors *in vivo*.
- Confirmed that, overall, high levels of GRP78, IL-11R and EphA5 expression were detected in lung cancer tumor specimens.
- Compiled a pre-IND package to be submitted to the FDA surrounding existing toxicology and efficacy data for a proapototic peptide targeting the IL-11R. This package includes extensive toxicology studies performed in non-human primates.

Project 4: Inhibition of bFGF Signaling for Lung Cancer Therapy

- Discovered that some NSCLC cells express both angiogenesis factors (bFGF and VEGF) and some of their receptors (FGFRs and VEGFRs). Furthermore, bFGF activated its receptor FGFR1 and the downstream pathways including ERK1/2 and Akt but not the PLCγ pathway. Although FGFR3 was constitutively activated it did not activate the same downstream pathways.
- Determined that bFGF acted as a mitogen in 1170-I cells by increasing the entry of cells from the G1 phase of the cell cycle to the S phase.
- Discovered the importance of the bFGF signaling in autocrine stimulation as indicated by the inhibition of cell growth in cells treated with siRNA targeting bFGF. Indirect evidence for this issue has come from the ability of inhibitors like TMPP and PD173074 to suppress the growth of 1170-I cells with and without bFGF stimulation.
- Determined that bFGF induced the migration and invasion of 1170-I cells.
- Discovered bFGF, FGFR1, and FGFR2 are frequently overexpressed in NSCLC in human lung tissue specimens, although different patterns of expression are detected in its two major types. Our findings further suggest that bFGF signaling pathway activation is an early event in the pathogenesis of squamous cell carcinoma of the lung. In addition, the frequent and early overexpression of bFGF and FGFR markers in patients with NSCLC suggests that the activation of the bFGF pathway, which has been proposed to facilitate the development of resistance to anti-angiogenic therapy targeting the vascular endothelial growth factor pathway, is an attractive novel target for lung cancer therapeutic and chemopreventive strategies.

Project 5: Targeting mTOR and Ras signaling pathways for lung cancer therapy

- Co-targeting of mTOR and PI3K/Akt signaling exerted enhanced anticancer activity in lung cancer xenograft models.
- Co-targeting of mTOR and PI3K/Akt signaling enhances inhibition of mTOR signaling while preventing Akt activation by inhibition of mTOR signaling.

Project 6: Identification and Evaluation of Molecular Markers in Non-Small Cell Lung Cancer (NSCLC)

- DNA profiling of 28 HNSCC matched T/N pairs and 14 matching lymph node metastases identified regions of LOH and allelic imbalance in HNSCC that are shared with adenocarcinomas.
- Genotyping of 8,430 tag SNPs from 24 genetically matched NAT and PT pairs and 12 matching lymph node metastases.
- Completed gene-specific hypermethylation analysis of 10 genes in HNSCC and NSCLC samples.
- Confirmed *TCF21* promoter hypermethylation and TCF21 expression as good biomarker of both early lung and head and neck cancer.
- Established 14 heterotransplant primary NSCLC tumor models, which will allow us to evaluate target therapeutic agents and to initiate biomarker discovery experiments.
- Identified dDNMT3B4 as regulator of RASSF1A promoter in human lung cancer cells.

Core B: Biostatistics & Data Management Core

- Continued to provide statistical support in the clinical trial design and revision for Project 1 and DRP-1.

- Provided data analysis for Projects 2, 3, 6, and Pathology Core.
- Continued to work closely with the Project 4 PI (Dr. Reuben Lotan) on synergy studies of combination drug treatment in cell lines to determine whether the effect is synergistic, additive, or antagonistic.
- Generalized and refined available methods to allow flexible modeling of drug interaction to account for the possibility that the combination may produce synergistic effect in certain dose ranges but additive or antagonistic in other dose ranges. The magnitude of drug interaction can also vary from dose to dose.
- Developed the code for implementing two new statistical methods – one parametric generalized response surface model and one semi-parametric model, which allow more general interaction patterns for the drug interaction and relax the restrictions of the existing methods.
- Developed methods to construct the confidence interval for the interaction index for the Emax model.
- Developed an additive hazards model with time-varying coefficients.
- Developed a new Bayesian cure rate model to estimate the cure rate and threshold.
- Developed a cure rate model with covariate measurement errors.
- Developed a dose-finding trial design with multiple drugs.

Core C: Pathology Core

- Developed a repository of lung cancer tissue, cytology and cell lines specimens with annotated clinical data, to be utilized for research projects and help to develop a series of lung cancer heterotransplants in mice in collaboration with Project 6.
- Characterized the expression of angiogenic markers (VEGF/VEGFR and bFGF/Receptors), HER family receptors, and tumor cell membrane/cytoplasmic proteins (GRP78, IL-11R and EphA5) in NSCLC, and establish their correlation with clinicopathologic features
- Identified the role of EGFR abnormalities in the progression and metastasis of lung adenocarcinomas, and establish the correlation with other important cancer pathways, such as ER/PR, NOTCH and NKX2-1.

Core D: Imaging Core

- Maintained SOPs and performed QC/QA procedures for routine synthesis of ^{111}In-DTPA-PEG-AnnexinV for SPECT/CT imaging in Project I (however, no imaging studies have yet been requested from the Imaging Core by investigators of this project).
- Produced novel ^{18}F-PEG6-IPQA derivative for PET and in vitro uptake studies for Project II;
- Performed all multi-modality imaging (and autoradiography) studies in mice with orthotopic models of different NSCLC using ^{18}F-PEG6-IPQA (EGFR expression/activity), ^{18}F-FMAU (proliferative activity), and ^{18}F-FDG (glucose metabolism) in Project II;
- Performed routine synthesis of [^{18}F]FEAU for Project III and conducted microPET imaging studies for this project.
- Performed synthesis and ^{111}In / ^{64}Cu radiolabeling of GRP78-specific cyclic nanopeptide CGRRAGGSC for imaging studies in Project III for imaging studies with ^{64}Cu-DOTA-CGRRAGGSC PET/CT in animals for Project III.
- CGMP production of precursor for 18F-radiolabeling of [^{18}F]-PEG6-IPQA has been subcontracted to CGMP qualified company (Macrocyclics, Inc., Dallas, TX) for the future Phase I clinical study.

DRP-1: Treatment of Malignant Pleural Effusion with ZD6474, a Novel VEGFR and EGFR TK Inhibitor

- Trial was approved and activated.
- Almost one third of the total sample size (25) has been enrolled.
- Collected 116 specimens for correlative analyses.

DRP-2: TALK - Teens and Young Adults Acquiring Lung Cancer Knowledge

- Protocol for testing videogame was approved and activated.
- Performed testing at two sites with a total of 303 participants.

CDP1: Identification of Membrane Proteins in Bronchial Epithelia Cells as Biomarkers of Early Detection for Lung Cancer

- Began organotypic cell culture for human bronchoepithelial cells and differentiation into squamous metaplasia.
- Optimized 2-D PAGE method.

REPORTABLE OUTCOMES

Resources

Database: We are generating an extensive database for targeting ligands and vascular receptors identified in our laboratory. This database is likely to be very useful as it can be integrated with the system that is in place under the IMPACT Program to correlate clinical information and responses to therapy with the expression of selective molecular targets.

Models: Additional 7 heterotransplant primary NSCLC tumor models have been established which allowed us to initiate evaluation of target therapeutic agents and biomarker discovery experiments. Three peer-reviewed publications were partially supported by this project.

Imaging Compounds: Radiolabeled compounds available for use by projects: ^{18}F-PEG6-IPQA (EGFR expression/activity), ^{18}F-FMAU (proliferative activity), and ^{18}F-FDG (glucose metabolism), and ^{111}In/ ^{64}Cu radiolabeling of GRP78-specific cyclic nanopeptide CGRRAGGSC.

Smoking Intervention Tool: The intervention tool is completed (kiosk and CD) and is currently being used for testing according to the study timeline. All educational modules are in place. The program underwent the first round of beta testing and all the glitches and problems were removed.

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1. Raso MG, Behrens C, Liu S, Prudkin L, Denise M. Woods, Natalie Ozburn, Cesar Moran, J. Jack Lee and Ignacio Wistuba. Immunohistochemical expression of estrogen and progesterone receptors identifies a subset of non-small cell lung cancers and correlates with EGFR mutations. Accepted for poster presentation in the 2008 AACR Annual Meeting (San Diego, CA, April 2008).
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adenocarcinoma of lung with worse prognosis.Accepted for platform presentation in the 2008 AACR Annual Meeting (San Diego, CA, April 2008).
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CONCLUSIONS

For the third year of the grant period, the research projects have been progressing as originally proposed in the grant with minor recommended changes indicated in grant year 2. Four clinical trials are opened and the final trial is in the IND development stage for an original imaging compound developed here.

This year, we have 10 publications, including 2 in *Nature* journals, 1 in *Clinical Cancer Research,* and 1 in *Journal of Clinical Oncology*, and 1 in Molecular and Cellular Biology. We have an additional 13 manuscripts accepted, in press, under review, submitted or in preparation, and 4 abstracts presented at 2007 AACR Annual meeting, 1 at the Society of Molecular Imaging, with an additional 4 submitted for this year's AACR Annual Meeting. Finally, we have created important integrated databases and 14 heteroptransplant models for long-term usage and cross-fertilization of other DoD Lung Cancer Research Programs.

In **summary**, the individual projects of IMPACT can conclude below:

Project 1: The combination targeted therapy (Tarceva) chemoradiation trial was activated and has begun accruing patients. Based on the preclinical work conducted to date, we are building a model to explain the molecular mechanism by which small molecule, tyrosine kinase inhibitors such as gefitinib and erlotinib suppress the cellular capacity for repair of DSBs thereby radiosensitizing NSCLC cells. Resistance to this mechanism may be due to compensatory pathways and these pathways can also be targeted with novel agents to restore radiosensitivity. Now that the animal studies and the clinical trial are underway, we will be able to validate that these same mechanisms are active and that these strategies are efficacious in vivo.

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Project 2: Several important findings have led us to the pre-IND phase with our lead compound identified. The [^{18}F]-PEG6-IPQA compound so far is the most PK optimized compound worthy of clinical translation. Imaging with pharmacokinetically optimized more water-soluble [^{18}F]-PEG6-IPQA (as opposed to [^{124}I]-mIPQA and [^{124}I]JGAP5 compounds) should allow for identification of tumors with increased EGFR signaling. The accumulation of [^{18}F]-PEG6-IPQA is highest in H3255 NSCLC cells that express L585R active mutant EGFR and correlates positively with the sensitivity of tumors to EGFR inhibitors (and is better than with [^{124}I]-mIPQA and [^{124}I]JGAP5). In addition, the accumulation of [^{18}F]-PEG6-IPQA can be observed to some extent in normal tissues that express EGFR (i.e., hair follicle cells), which are currently used as surrogate biomarkers of EGFR activity/inhibition and which represents additional proof of the approach to imaging EGFR kinase activity with [^{18}F]-PEG6-IPQA.

Project 3: We continued with preclinical development of candidate ephrin mimics (GGS peptides) targeting these lung cancer cells moving quickly towards an IND and development of

a clinical trial using this novel target and agents.

Project 4: We continued our research on bFGF delineating the sequence of molecular events in NSCLC. Our findings this year expand and confirm the data on the mitogenic effect on NSCLC cell lines in a time- and dose-dependent fashion by acting in the transition from the G1 to S phases of the cell cycle. We have also evaluated the bFGF inhibitors TMPP and PD173074 and demonstrated growth suppression in NSCLC cell lines.

Project 5: Targeting the mTOR axis appears to be a promising strategy against lung cancer. Given the nature of the complexity of lung cancer signaling pathways including mTOR signaling, it is essential to understand the biology of lung cancer and the action mechanism of the interested therapeutics in order to efficiently treat lung cancer through application of mechanism-driven therapeutic regimens. We continued our studies using xeongraft models, demonstrating that co-targeting mTOR and PI3K/Akt signaling enhances anticancer efficacy. We also launched our clinical trial for RAD001 and have begun accruing patients.

Project 6: We confirmed *TCF21* promoter hypermethylation and its potential use as an biomarker in both HNSCC and NSCLC. With an established role in the epithelial-mesenchymal transition, *TCF21* may be important in predicting metastatic potential. We have continued our interrogation and profiling utilizing our matched tumor/normal pairs and matching lymph node metastases as well as our heterotransplant primary NSCLC models. Finally, we have identified a new ΔDNMT3B family member important in regulation of promoter methylation in lung cancer.

Biostatistics Core: Core B continued to provide statistical and data management support for all research projects in the IMPACT study and develop new methods of analyses.

Pathology Core: The Pathology Core has assisted and collaborated actively with several research projects performing multiple histopathological, immunohistochemical and molecular studies in a large series of lung cancer tissues specimens. In addition, the Pathology Core has managed to conduct specific research activities, which fully integrate with some of the IMPACT research projects.

Imaging Core: The Imaging Core continues to provide imaging support requested from the PIs of IMPACT research projects in addition to synthesizing more optimal IPQA derivatives.

DRP-1: The clinical trial is well underway; all analyses will be performed after the trial is completed.

DRP-2: Project TALK is successfully developing according to the timeline. It produced an innovative, highly informative, and easy-to-navigate videogame which was enthusiastically accepted by young individuals involved in the pre-testing activities. The investigative team has begun testing and has moved to the evaluation phase of the study for which community venues have been identified and their support and cooperation secured. A preliminary analysis is expected in March 2008.

CDP1: This career development project has just begun. It has enormous potential for interesting proteomic results on tumor development which may be developed into early screening tools as well as for training and career development.

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Appendices